INVESTIGATING THE MOLECULAR BASIS OF ZN AND CD TRANSPORT AND HOMEOSTASIS IN THE METAL HYPERACCUMULATING PLANT SPECIES, *THLASPI CAERULESCENS*

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INVESTIGATING THE MOLECULAR BASIS OF ZN AND CD TRANSPORT AND HOMEOSTASIS IN THE METAL HYPERACCUMULATING PLANT SPECIES, *THLASPI CAERULESCENS*

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Thlaspi caerulescens (J&C Presl) is a Zn/Cd hyperaccumulator which tolerates Zn and Cd toxic soil environments and accumulates both metals in the shoot to extremely high levels without any toxicity symptoms (30,000 ppm Zn and 12,000 ppm Cd on a leaf dry weight basis). This ability to hyperaccumulate heavy metals has intrigued plant biologists for many years for the possible value *T. caerulescens* may have in the phytoremediation of Zn- and Cd-contaminated soils. However, it's slow growth and diminished shoot biomass limits its usefulness for phytoremediation. Exploiting the genetic potential of this species by transferring metal hyperaccumulating traits to plants with a higher biomass may be an effective approach to generate new and novel metal accumulator plants. To accomplish this goal, a better understanding of the basic molecular and physiological mechanisms responsible for Zn/Cd hyperaccumulation is needed. Also, because we had previously shown that Zn hyperaccumulation in *T. caerulescens* is related to the elevated or altered Zn-dependent expression of a number of metal transport and metal-related genes, this plant may serve as a useful tool for studying plant mechanisms of Zn sensing and homeostasis.

In an attempt to better understand how the transport of Zn and Cd from the soil to the shoot is altered in metal hyperaccumulators, *T. caerulescens*

was compared in this study to two closely related nonaccumulator plants, *Thlaspi arvense* and *Arabidopsis thaliana*. Additionally ecotypic variation within *T. caerulescens* was exploited. These two approaches were used to identify key proteins involved in Zn and Cd uptake, regulation and sequestration. One component of the research was the functional characterization of the micronutrient transport TcZNT1, which had previously been suggested to be the root Zn/Cd uptake transporter in *T. caerulescens*. TcZNT1 was characterized in comparison to its closest sequence-based homolog in Arabidopsis, AtZIP4. From this research we obtained findings that TcZNT1 most likely is involved in root Zn uptake from the soil, but also found a possible new role for this transporter in long distance Zn transport based on high levels of *TcZNT1* gene expression in the root and leaf vasculature. While it was found that AtZIP4 shares a number of similarities to TcZNT1 with regards to metal transport and tissue-specific expression, we identified a major difference in that AtZIP4 is localized to the chloroplast while TcZNT1 is a plasma membrane transporter. Hence AtZIP4 must play a role in chloroplast micronutrient homeostasis. A second component of the research focused on potential transcriptional regulators of *T. caerulescens* Zn transporter genes. Using yeast complementation assays, we identified intriguing candidates for this role based on the ability of *T. caerulescens* transcription factors to activate the expression of a yeast high affinity Zn uptake transporter. These proteins are members of the E2F family of transcription factors that are thought to be involved solely in cell cycle regulation. Here it was shown that one of these TcE2Fs binds with high affinity to a putative E2F element in the TcZNT1 promoter. TcZNT1 is closely related in sequence to the yeast Zn transporter gene that was activated by the TcE2F. However, to date, we have not shown

direct activation of *TcZNT1* expression by TcE2F2. The final component of this research project exploited natural ecotypic variation in Cd hyperaccumulation between two *T. caerulescens* ecotypes, and used a comparative transcriptomics approach between these ecotypes to identify a candidate transporter for the enhanced Cd accumulation in the one ecotype. This transporter, TcHMA3, is a member of the P-type ATPase family of metal transporters and was found to be a vacuolar Cd transporter expressed in both the roots and shoots of *T. caerulescens*. Overexpression of this vacuolar transporter in transgenic Arabidopsis led to increased Cd tolerance and increased root Cd accumulation.

BIOGRAPHICAL SKETCH

Matthew John Milner was born on May 31st 1981 to the proud parents of John Milner and Mary Frances Picciano Milner in Champaign Il. Matthew lived the normal childhood of kid growing up surban America. With an older sister Kristina to terrorize him and keep him in line he managed to grow up and finish high school in 1999 from State College Area High School. Matthew decided to attend Pennsylvania State University in the Fall of 2000 and major in Biology with an emphasis on plants. In December of 2004 Matthew graduated from Pennsylvania State University and eventually contacted Dr. Leon Kochian about working in his lab for a few months. Those few months turned into six years and eventually lead to this dissertation. What the future holds no one knows but I am excited to find out.

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This is a large THANK YOU to all of those involved in helping me get this far. This includes Leon, for taking a chance on an overzealous student. My parents, John and Mary Frances for bringing me into this world and raising me to be the man I am today. My sister for using me as a test subject for all of the rumors she heard growing up. My friends who helped me realize there is more to life then work. My girlfriend for pointing out that I do not have a Science paper yet. My puppy Borlog who distracted me from writing this dissertation. Members of the Kochian lab, John and Eric who listen to me babble on about nothing most days. Former members Nicole, Ashot and Melinda who helped me learn the tools to succeed in the lab. And the rest of the Kochian lab for guidance and support.

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INVESTIGATING HEAVY METAL HYPERACCUMULATION USING *THLASPI CAERULESCENS* AS A MODEL SYSTEM: A LITERATURE REVIEW

Introduction:

The ability of terrestrial plants to deal with a broad array of abiotic conditions has allowed certain plant species to adapt to extremely harsh environments. One of the potential abiotic stresses a plant may face is toxic levels of heavy metals in the soil. The typical terrestrial plant has a limited capacity for dealing with excess metals; the main approach that most plants use to deal with heavy metals is storage in the root cell wall and vacuole, thus keeping the heavy metal sequestered from the root cytoplasm and more importantly, from long distance transport from the root to shoot. However there are a number of plant species that have evolved on metalliferous soils and thus are adapted to extreme soil metal environments. Currently there are believed to be around 400 plant species from a number of different families such as the Asteraceace, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae, and Fabaceae that possess the ability to tolerate very high levels of heavy metals in the soil and more importantly, in the plant shoot. The Brassicaceae is the best represented amongst these metal hyperaccumulator families with 87 Brassica species classified as metal hyperaccumulators. Hyperaccumulation was a term first coined by Brooks *et al*. (1977) for plants that are endemic to metalliferous soils and are able to tolerate and accumulate metals in their above ground tissues to very high concentrations (~100 times that of a nonaccumulator plant species). Of these 87 different species in the Brassicaceae, two species in particular, *Thlaspi caerulescens* and *Arabidopsis*

halleri, have been studied extensively for their ability to hyperaccumulate several heavy metals, mainly Zn, Cd, and Ni. In particular, certain ecotypes of *T. caerulescens* can accumulate as much as 30,000 ppm of Zn and approximately 10,000 ppm Cd in the shoot biomass without any signs of toxicity (typical shoot levels are 100-200 ppm Zn and 0.1 to 10 ppm Cd).

Contamination of soils with heavy metals is both an environmental problem as well as a risk to human health (Gairola *et al*., 1992; Mazess and Barden, 1991 Ryan *et al*., 1982). The remediation of metal-contaminated soils based on relatively destructive engineering-based methods has been estimated to cost approximately 400 billion dollars in the US alone (Salt *et al*., 1995). Thus there has been interest in recent years in determining whether an understanding of the mechanisms that metal hyperaccumulator plant species such as *T. caerulescens* employ to absorb, detoxify and store metals can be used to develop plants suited for the remediation of metal contaminated soils via phytoremediation.

Over the past ten years as interest in metal hyperaccumulating plants has increased, *T. caerulescens* has been at the forefront of studies concerning the hyperaccumulation process. Because *T. caerulescens* is a slow growing plant species that does not generate significant shoot biomass, it has been used primarily as a model system for the investigation and identification of the underlying molecular and physiological mechanisms of hyperaccumulation, with the ultimate goal of transferring these mechanisms to higher biomass plant species. In this chapter, an overview of the physiological mechanisms of heavy metal hyperaccumulation as well as the underlying molecular and genetic determinants for this trait will be discussed, with a focus on the body of work with *T. caerulescens*.

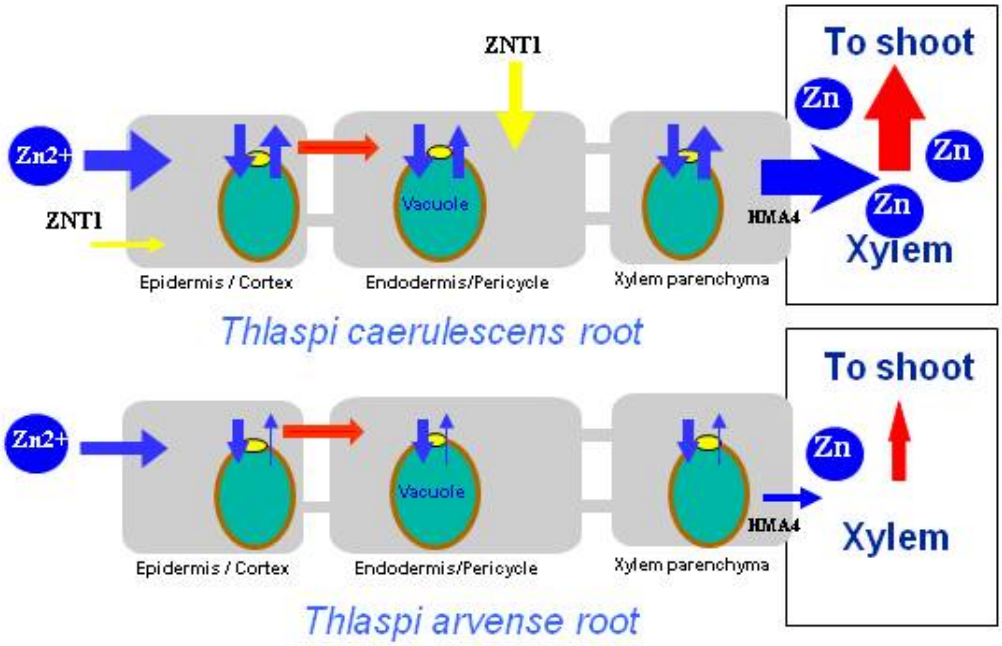
***Physiology of Metal Hyperaccumulation in Thlaspi caerulescens*:**

The metal transport component of hyperaccumulation in *T. caerulescens* appears to involve at least four physiological events. The first is a stimulated metal influx across the root cell plasma membrane, the second reduced metal sequestration in the root vacuole, the third increased loading into the xylem for transport to the shoots, and finally, the fourth involves stimulated metal influx across the leaf cell plasma membrane and sequestration in the leaf vacuole. These transport steps resulting in metal hyperaccumulation are summarized in the model depicted in Figure 1.1 for transport in and across the root, and Figure 1.2 for transport in the leaf.

Based on a detailed concentration-dependent root Zn uptake kinetic analysis for *T. caerulescens* compared with a closely related non-accumulator species, *T. arvense*, it was found that the affinity of the root Zn transporter for Zn was similar between the two species (K_m values of 6 μM for *T. caerulescens* and 8 μM for *T. arvense*), however the maximal Zn uptake (V_{max}) in *T. caerulescens* was found to be approximately six times higher (Laset *et al*., 1996). This suggests that the Zn transporters in roots of *T. caerulescens* are not more efficient at transporting Zn than in non-accumulator plants; instead, it appears that the density of Zn transporters in the root-cell plasma membrane is much higher in *T. caerulescens* versus *T. arvense*. In both *Thlaspi* species, root Zn uptake appears to be regulated by plant Zn status. In *T. arvense*, like other non-accumulators, root Zn uptake increases as the plant transitions from Zn sufficiency to deficiency. *T. caerulescens*, on the other hand, maintains its much greater root Zn influx in both Zn deficient and sufficient conditions. Only when *T. caerulescens* is grown on very high Zn concentrations (nutrient solution with 50 to 1000 μM Zn) does the V_{max} for Zn

Figure 1.1: Model of Zn transport into and across the root of *T. caerulescens*. The model depicts some of the aspects of and differences in the movement of Zn^{2+} from the soil into and across the root of *T. caerulescens* compared with *T. arvense*, with several possible candidate transporters indicated at specific transport steps. For uptake of Zn from the soil into the root epidermis/cortex, *T. caerulescens* exhibits elevated levels of influx across the root-cell plasma membrane compared with *T. arvense*. Previous speculation in the literature suggested TcZNT1 may be the transporter facilitating this uptake, but recent localization of the Arabidopsis homolog of the *TcZNT1* gene suggests it may actually be more involved in metal loading into the stele. The model also indicates less vacuolar sequestration of Zn in roots of *T. caerulescens*, which would keep more of the absorbed Zn in a mobile pool moving radially through the endodermis and pericycle to the xylem parenchyma. Zn is then believed to be loaded into the xylem vessel elements via the ATPase, HMA4, for subsequent transport to the shoots.

Zn transport into and across the root



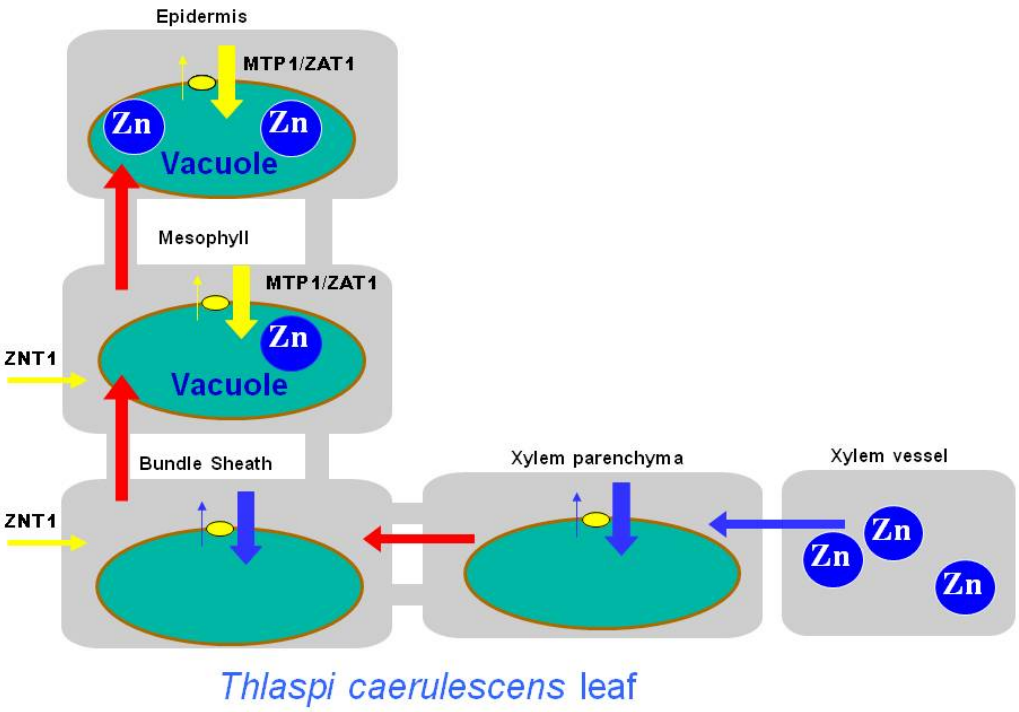


Figure 1.2: Model of Zn transport and storage in the leaf. The model depicts Zn transport in the leaf from the xylem vessel through xylem parenchyma and bundle sheath cells to the sites of leaf storage, the leaf mesophyll and epidermis. Based on cellular expression studies in leaves of *T. caerulescens*, we suggest ZNT1 may be the transporter facilitating uptake into bundle sheath and mesophyll cells. How Zn ultimately is loaded into epidermal cells is not known. Here we suggest that the transporter, MTP1/ZAT1, may facilitate vacuolar sequestration of metals in mesophyll and epidermal cells.

uptake decrease, but is still maintained at flux values higher than what is seen in *T. arvense*. Another interesting aspect of the relationship between plant metal status and accumulation in *T. caerulescens* comes from the work of Papoyan *et al.* (2007). In this study, it was found that when *T. caerulescens* was grown on very high Zn levels, the plants were more Cd tolerant and accumulated more Cd. The converse was also true, in that high Cd-grown plants also accumulated more Zn than low Cd-grown plants. This stimulated shoot metal accumulation was associated with enhanced root metal influx, and xylem transport of these metals from the root to shoot. The authors speculated that as xylem loading is a key step in the hyperaccumulation process, the enhanced xylem loading triggered by exposure to high heavy metal levels for extended periods, may translate into improved heavy metal tolerance, as the metals are more efficiently translocated to the shoots where highly effective metal tolerance mechanisms operate. When all of these studies are considered together, it appears that the regulation of expression of micronutrient/heavy metal transporters by plant metal status is altered in an unknown fashion in the hyperaccumulating Thlaspi species, resulting in higher expression of several different transporters along the metal transport pathway from the soil to the shoot. Once the Zn enters the root, the plant deals with the Zn in two ways: either by storing Zn in the root vacuole or by transporting the Zn radially through the root to be loaded into the xylem for transport to the shoots. Root Zn compartmentation via efflux analysis was studied in both *Thlaspi* species (Lasat *et al*., 1998). It was found that *T. arvense* stored approximately 2.5 times more Zn in the root vacuole compared to *T. caerulescens* and this vacuolar Zn was released from the vacuole twice as slowly in *T. arvense*. Longer-term studies showed that as much as six times

as much Zn was sequestered in the root-cell vacuoles of *T. arvense* compared to *T. caerulescens* over a 46-hour time period (Lasat *et al*., 1998). These findings indicate that along with much higher rates of Zn entry into the root, the hyperaccumulator species also maintains the root Zn in a more mobile pool that is moved to the xylem much more readily.

The next key step regarding the movement of heavy metals from the soil to the shoot is the loading of Zn from xylem parenchyma into xylem vessels for translocation to the shoot. Xylem sap from *T. caerulescens* was found to contain approximately a five fold higher Zn concentration compared to the xylem sap from *T. arvense* for plants grown on the same Zn level. While the Zn concentration in the xylem increased with increased external concentrations of Zn, the ratio of xylem sap Zn concentration between the two *Thlaspi* species stayed the same (Lasat *et al*., 1998). The dramatically greater metal loading into the xylem is a hallmark of metal hyperaccumulators and may be due to the activity of a P-type ATPase, HMA4, which will be discussed in greater detail in the molecular studies section later in this chapter. These root-associated transport steps are summarized in the model depicted in Figure 1.1.

In the shoot, the storage of Zn and Cd to very high levels appears to require some coordination between different cell types. The highest concentrations of Zn and Cd are found in leaf epidermal cells, with concentrations four times that of mesophyll cells (Küpper *et al*., 1999). This preferred storage in leaf epidermal cells may be associated with avoidance of heavy metal damage to photosynthesis, as epidermal cells (except for guard cells) lack chloroplasts. Although these metals are accumulated to much higher levels in the epidermis, a significant fraction of the total leaf metal

accumulation still occurs in the mesophyll, as a larger fraction of the leaf biomass is associated with the mesophyll. It has been calculated that 65-70% of the total Zn in the leaves is in the mesophyll (Ma *et al*., 2005). The transport steps for metal movement in the leaf, which is more poorly understood than root metal transport in *T. caerulescens*, are summarized in the model depicted in Figure 1.2.

It appears as if every cell in *T. caerulescens* shows a high level of tolerance to Zn and not just the cells and tissues where the metal is ultimately stored. Using suspension cells cultures of *T. caerulescens* compared with *Arabidopsis* suspension cells used as representative of normal, non-accumulator plant cells, it was found that at the cellular level, *T. caerulescens* suspension cells were indeed more tolerant to high levels of Zn and Cd than *Arabidopsis* suspension cells (Klein et al., 2008). However, it was interesting that the *T. caerulescens* cell lines accumulated less Zn and Cd than the Arabidopsis cell lines, most likely due to a greater metal efflux. This finding led the authors to hypothesize that the *T. caerulescens* suspension cells represent cells of the Zn/Cd transport pathway between the root epidermis and leaf, which appear to function to keep the Zn/Cd in a mobile pool that is readily translocated to the shoot.

To better dissect and further understand the role that the roots and shoots both play in the Zn/Cd hyperaccumulation phenotype, a recent study using reciprocal grafting between *T. caerulescens* and a closely related non accumulator, *T. perfoliatum*, was performed to study the role of roots and shoots on the ability of *T. caerulescens* seedlings to hyperaccumulate Zn (Guimarães et al., 2009). In this paper it was shown that when *T. perfoliatum* root stock was grafted to a shoot scion of *T. caerulescens*, the grafted shoot

no longer hyperaccumulated Zn, but rather accumulated Zn to levels seen in *T. perfoliatum* shoots. However when the reciprocal graft was made, with *T. perfoliatum* shoots on *T. caerulescens* roots, the *T. perfoliatum* shoots hyperaccumulated Zn when the grafted seedlings were grown on lower Zn levels. This ability to accumulate large levels of Zn in the shoots was reduced at higher Zn levels, as Zn toxicity started to occur, indicating that other tolerance mechanisms must be operating in the shoots of *T. caerulescens* compared with *T. perfoliatum*.

It is presumed that an important aspect of metal storage involves metal chelation with organic ligands. However, it is still not clear which specific ligands are involved. Salt *et al*., (1999), using X-ray absorption spectroscopy, obtained results indicating that the form of Zn in *T. caerulescens* differed depending on the location in the plant. It was suggested that a significant fraction of the Zn within the roots is associated with histidine, in the xylem sap Zn was mostly found as the free hydrated Zn^{2+} ion with a small portion bound to organic acids, and in the shoots Zn was most commonly found associated with organic acids, with smaller fractions found as the free ion and bound to histidine or the cell wall. It would be expected that metals such as Zn and Cd would be associated with sulfur ligands such as those in cysteine, glutathione, or phytochelatins. Küpper *et al*. (2004), based on x-ray absorption spectroscopy analysis, suggested that a large portion of the Cd in the leaves of *T. caerulescens* was bound to sulfur ligands but most of the Cd was bound to oxygen molecules, most likely in the form of organic acids. Also, using ^{113}Cd NMR, Ueno *et al*. (2005) presented findings suggesting a significant portion of the leaf Cd was bound to organic acids, mainly malate. Malate is not a particularly strong ligand for Cd, thus Ueno *et al*. (2005) speculated that

efficient transport of Cd into the vacuole, which already contains fairly high malate concentrations, would facilitate the malate-Cd interaction. While there were already large amounts of malate in the vacuole, Cd treatment did not facilitate any increases in leaf malate levels.

It would be expected that phytochelatins (PC), that are believed to play a role in plant Cd tolerance, would be involved in Cd hyperaccumulation in *T. caerulescens*. However, in a study comparing phytochelatin levels in leaves of *T. caerulescens* and *T. arvense* when plants were exposed to high Cd levels, no differences were found in leaf or root phytochelatin levels in these two plant species (Ebbs *et al*., 2002). A recent study looking at the effect of inhibition of PC biosynthesis on Cd tolerance showed that the *T. caerulescens* ecotype, Prayon, exhibited an increased sensitivity to Cd stress when a key enzyme in the phytochelatin synthetic pathway, γ-glutamylcysteine synthetase (γ-ECS), was inhibited, suggesting that phytochelatins may play at least an initial role in Cd tolerance (Hernàdez-Allica *et al*., 2006). It is interesting to note that in the same study, the Ganges ecotype, which accumulates considerably more shoot Cd than other *T. caerulescens* ecotypes (see discussion below), showed no change in Cd tolerance when γ-ECS was inhibited, suggesting Ganges may employ a different Cd tolerance mechanism than other *T. caerulescens* ecotypes. The authors speculated that a low molecular weight thiol other than phytochelatins that has not yet been identified might play a role as a Cd ligand in the Ganges ecotype.

As mentioned above, *T. caerulescens* ecotypes from the south of France, such as Ganges, accumulate much more leaf Cd than other ecotypes (with Prayon being the most widely studied “other” ecotype), while all of the ecotypes are approximately equal in their ability to hyperaccumulate Zn. A

detailed kinetic analysis of root Cd and Zn influx showed there was an approximately 5 fold larger V_{max} for root Cd uptake in Ganges versus Prayon in short-term radiotracer studies, while the K_m value for Cd uptake was not different (Lombi *et al*., 2001). Comparison of Zn influx between the two ecotypes showed no differences in either V_{max} or K_m (Lombi *et al*., 2001). These physiological studies suggested there is a high affinity Cd uptake transporter in the roots of the Ganges ecotype that is not present in Prayon, which contributes to the enhanced Cd hyperaccumulation in Ganges. It is not clear what the identity of this Cd transporter is. However, in a subsequent study, Lombi and coworkers (Lombi *et al*., 2002) showed that the imposition of Fe deficiency on the two ecotypes specifically induced high affinity root Cd uptake in Ganges. They also found that the root Fe uptake transporter IRT1 that has also been shown to transport Cd (Vert *et al*., 2002; Connolly *et al*., 2002; Cohen *et al*., 2004), was induced by Fe deficiency in roots of Ganges but not Prayon. These findings are described in more detail in the section on the molecular biology of metal hyperaccumulation.

Field and Soil Studies:

Some interesting field studies have been performed over the past several years that may indicate that under certain conditions, despite its low biomass production, it might be possible to use *T. caerulescens* for the phytoremediation of certain metal contaminated soils. It appears that variables such as season length, method of sowing seed, and soil pH have effects on the Zn and Cd extraction capabilities of *T. caerulescens* from the soil (McGrath *et al*., 2006; Yanai *et al*., 2006). It was found that a soil pH of between 5 and 6 seems to be optimum to facilitate Cd extraction as well as

plant biomass production (Yanai *et al*., 2006). Also leaving the plants over winter increased the biomass production almost 5 fold and also increased the amount of Zn taken up by 57 % versus that of a single four-month growing period (McGrath *et al*., 2006). There was no difference seen in Cd accumulation between the four and 14-month growth conditions. In terms of the sowing of *T. caerulescens* plants, there was a greater than two fold increase in the Cd and Zn taken up from the soil when the plants were sown as seedlings from a subplot versus that of planting seeds directly into the soil (McGrath *et al*., 2006). These few studies indicate that more agronomically-based research on the practical aspects of metal hyperaccumulation and phytoremediation is needed.

With regards to metal hyperaccumulation from the soil, it has been suggested in earlier studies that *T. caerulescens* may be able to mine the soil for metals more efficiently than non-accumulator plants (Knight *et al*., 1997; McGrath *et al*., 1997). That is, it may be that *T. caerulescens* may have access to a different, more tightly-bound pool of soil metals than non-accumulating plants, and it has been speculated that this may involve root organic acid exudation as a means of increasing metal availability in the rhizosphere. However, it should be mentioned that in hydroponic studies, where all the Zn and Cd is available, *T. caerulescens* still accumulates much more Zn and Cd than *T. arvense*, even at low Zn/Cd concentrations (Lasat *et al*., 1996; Pence *et al*., 2000). There have been a few studies suggesting that rhizosphere metal availability plays a role in metal hyperaccumulation. For example, when *T. caerulescens* was grown in axenic versus non-sterile soil, the axenically grown plants accumulated 25-42% less Zn in the shoots, suggesting that rhizosphere microbes may play a role in metal availability for

the hyperaccumulator (Whiting *et al*., 2001). Also, Ingwersen *et al*. (2006) found that *T. caerulescens* can increase the availability of extractable Cd in the rhizosphere primarily through the kinetically limited desorption of Cd from soil particles. However, a number of other studies did not find that *T. caerulescens* has any special properties with regards to mining the soil for heavy metals. Hammer *et al*. (2006) found the *T. caerulescens* did not accumulate more metal due to an increased pool of metals in the soil solution as has been suggested above. They found that *T. caerulescens* accessed the same soil metal pools as that of the non-accumulator, *Brassica napus*, under the same conditions. With regards to root exudation of organic acids, Zhao *et al*. (2001) found no significant differences in organic acid exudation from the roots of *T. caerulescens* compared with *T. arvense*. Based on the evidence that *T. caerulescens* has the same abilities as non-accumulators with regards to increasing metal availability in the rhizosphere, it was then suggested that it might be possible to amend the soil with materials such as EDTA or citrate to increase metal solubility, and facilitate better metal extraction by *T. caerulescens*. However, McGrath *et al*. (2006) found that the addition of certain amendments such as EDTA may actually decrease biomass production of the hyperaccumulators, thus reducing their effectiveness, possibly by increasing the solubility of metals such as Cu to levels that are toxic to the hyperaccumulator. A number of other possible amendments such as NTA and citric acid were also looked at under field conditions and seemed to have no effect on increasing metal content or growth of *T. caerulescens* (McGrath *et al*., 2006).

Genetic Analysis of Metal Hyperaccumulation:

A recent review of the Brassica family as a whole investigated various factors such as lifecycle, self-incompatibility, genetic resources and plant size to help find the best species within this family in order to begin to investigate the genetic basis for both Ni and Zn hyperaccumulation (Peer *et* al., 2006). To date, only a few genetic resources such as mapping or diversity populations have been developed for *T. caerulescens*, and only very recently have the first studies, which focused on QTL analysis of metal accumulation, been published.

The first QTL analysis study involved the phenotyping of an F2 population generated from a cross between a higher Zn accumulating *T. caerulescens* ecotype from a non-metalliferous location [Lellingen (LE)], and a relatively lower Zn accumulating ecotype from a calamine soil [La Calamine (LC)]. Two major QTL were found for increased root Zn accumulation, with each parent in the population contributing one of the QTL. The two QTL explained 22% and 17% of the variation in root Zn accumulation, respectively (Assunção *et al*., 2006). The second publication looked at QTL for both root and shoot Cd and Zn accumulation for a mapping population from a parent from a Pb/Cd/Zn-contaminated site near La Calamine, Belgium, while the second parent was selected from a site with similar soil characteristics near Ganges, France (Deniau *et al*., 2006). The authors identified eight total QTL between the two populations, with the Ganges ecotype contributing six of the QTL. Two QTL for root Cd accumulation and two for root Zn accumulation were identified, as well as three shoot Zn accumulation QTL and one for shoot Cd accumulation. These QTL explained 24% of the variation in Zn content and 60% of the variation in Cd content within the population. It is interesting to

note that there was overlap of QTL for root Zn and Cd accumulation as well as for shoot Zn and Cd accumulation. Obviously the genetic analysis of the metal hyperaccumulation trait is in its infancy, but these studies suggest that metal hyperaccumulation in *T. caerulescens* is a relatively complex, quantitative trait.

Molecular Biology of Metal Hyperaccumulation in T. caerulescens:

Molecular analysis of metal transporters: Major advances in the identification of a number of plant micronutrient/heavy metal transporter families has occurred, and new transporters such as the ZIP (ZRT/IRT like Protein), CDF (Cation Diffusion Facilitator), Nramp (Natural Resistance and Macrophage Protein), and HMA (Heavy Metal ATPase) families have been recently identified, primarily in the model plant species *A. thaliana*. However to date only a handful of these transporters have been identified and characterized in *T. caerulescens.* A brief summary for each of the transporters discussed in this section is listed in Table 1.1.

The first transporter gene cloned from *T. caerulescens* was *ZNT1* by Pence *et al*. (2000). *TcZNT1* is a member of the ZIP family of transport proteins. The first members of this family were identified based on homology to the high affinity Zn uptake transporter in yeast, ZRT1, and the Arabidopsis Fe transporter, IRT1. TcZNT1 is a putative plasma membrane-localized transporter that can mediate Zn and Cd uptake when expressed in yeast (Pence *et al.*, 2000). *TcZNT1* shares high homology with *ZRT1*, the yeast high affinity Zn uptake transporter, as well as *AtZIP4* from *Arabidopsis thaliana*. It was found that changes in root *TcZNT1* expression induced by alterations in plant Zn status closely paralleled changes in the kinetics of root Zn uptake, leading the authors to speculate that TcZNT1 may be a major

transporter mediating root Zn and Cd uptake from the soil (Pence *et al.*, 2000). One of the hallmarks regarding expression of this gene in *T. caerulescens* versus expression of its homolog in non-accumulators such as *T. arvense* and *A. thaliana* is that it is expressed at much higher levels in roots and shoots of *T*. *caerulescens*.

Furthermore, the response of *TcZNT1* expression to changes in plant Zn status is different than in non-accumulator plants. As seen in Figure 3, *TaZNT1* is expressed at very low levels in roots and shoots of a non-accumulator plant (*T. arvense*) grown on sufficient (1 μM) and high (10 μM) Zn. However, when these plants were made Zn deficient, *TaZNT1* expression increased, presumably to synthesize more Zn transporters to facilitate Zn uptake. The same pattern is true for the Arabidopsis homolog, *AtZIP4* (Grotz *et al*., 1998). In *T. caerulescens*, expression of *TcZNT1* is very high in both Zn deficient and sufficient (1 μM Zn-grown) plants. Because *T. caerulescens* is relatively Zn inefficient, and requires higher Zn levels to achieve Zn sufficiency (Levent *et al*., 2003), it may be that growth on 1 μM Zn may not yield totally Zn sufficient plants (although there are no observable symptoms of Zn deficiency in these plants). Or, when grown on this particular Zn concentration, the plants may behave as though they are physiologically Zn deficient as the metal is being effectively tied up as part of the hyperaccumulation phenotype. When *T. caerulescens* plants are grown at very high Zn levels (50 – 1000 μM), one sees a significant down regulation of *TcZNT1* expression (Figure 3). However, even at these very high Zn levels, *TcZNT1* expression is still significantly higher than that of its homologs in *T. arvense* or Arabidopsis. Thus it has been suggested that hyperexpression of *TcZNT1* and other metal-related genes involves an alteration in the regulation of these genes by plant metal

Table 1.1: Summary of the transporters identified to date in the Zn/Cd hyperaccumulating species, *T. caerulescens*, and the related model species, *A. thaliana.* Transporters are organized by gene family, a brief description of the putative function is presented, as well as the plant tissues in which each gene is expressed.

Family	Summary	Tissues Expressed
ZIP Family		
TcZNT1	High affinity Zn transporter in *T. caerulescens*	Mainly Roots
AtZIP4	Cu transporter in *A. thaliana*	Mainly Roots
AtIRT1	High affinity Fe transporter	Mainly Roots
TcIRT1	High affinity Fe transporter	Mainly Roots
ZRT1	High affinity Zn transporter in *S. cerevisiae*	
CDF Family		
AtMTP1/ZAP1	Zn tonoplast transporter	Mainly Shoots
TgMTP1	Ni plasma membrane transporter	Mainly Shoots
TcMTP1/ZTP1	Putative Zn tonoplast transporter	Mainly Shoots
HMA Family		
TcHMA4	Zn/Cd efflux transporter involved in loading into the xylem	Roots and Shoots
AtHMA2	Zn/Cd efflux transporter involved in loading into the xylem	Roots and Shoots
AtHMA4	Zn/Cd efflux transporter involved in loading into the xylem	Roots and Shoots
AhHMA4	Zn/Cd efflux transporter involved in loading into the xylem	Roots and Shoots
Metallothionein Family		
TcMT1	Metal ligand that increases tolerance to Cu, Zn and Cd	Shoots
TcMT2	Metal ligand that increases tolerance to Cu, Zn and Cd	Roots and Shoots
TcMT3	Metal ligand induced by Cd involved in Cu homeostasis	More Highly in the Shoots
AtMT1	Metal ligand that increases tolerance to Cu, Zn and Cd	Shoots
AtMT2	Metal ligand that increases tolerance to Cu, Zn and Cd	Shoots
Nicotianamine Synthase Family		
TcNAS1	Nicotianamine producing enzyme believed to be involved in Ni tolerance	Mainly Shoots
Yellow Stripe Like Family		
TcYSL3	Ni-NA and Fe-NA transporter	Roots and Shoots
TcYSL5	Putative metal-NA transporter	Shoots
TcYSL7	Putative metal-NA transporter	Roots and Flowers

status, and this hyperexpression may play a role in hyperaccumulation (Pence, 2002; Letham *et al.*, 2005).

It is interesting to note that although in Arabidopsis, *AtZIP4* is the closest homolog to *TcZNT1*, sharing 90% DNA sequence identity as well as 81% homology at the amino acid level, it may function as a different metal transporter. *TcZNT1* was cloned via complementation of yeast mutants defective in endogenous Zn transporters (the *zrt1/zrt2* mutant) and thus could not grow on low Zn (Pence *et al*., 2000). *TcZNT1*, at least in yeast, was shown to be a high affinity Zn uptake transporter and low affinity Cd uptake transporter. However, Grotz et al. (1998) showed that *AtZIP4* could not complement the same *zrt1/zrt2* Zn uptake deficient yeast mutant. *AtZIP4* was later shown to complement the *ctr1* yeast mutant, which is defective in Cu uptake, thus allowing the mutant to grow under Cu limiting conditions (Wintz *et al*., 2003). These findings suggest that AtZIP4 is involved in Cu uptake and not Zn/Cd uptake. Furthermore, *AtZIP4* transcript levels were shown to increase not only by Zn deficiency, but also by Cu deficiency (Wintz *et al*., 2003). Furthermore, the cell-specific expression of these transporters also may suggest they have different roles than initially suggested. As mentioned above, metal accumulation in the *T. caerulescens* leaf is much higher in epidermal cells (except guard cells) compared with other leaf types. However, Küpper *et al.* (2007), using a quantitative in situ hybridization technique, found that *TcZNT1* is not expressed in the leaf epidermal cells. Instead, it is preferentially expressed in leaf mesophyll, bundle sheath and guard cells, leading them to postulate it plays a role in normal leaf Zn nutrition and not metal hyperaccumulation in *T. caerulescens*.

The second gene cloned from *T. caerulescens* was *ZTP1/MTP1*, which was identified based on homology to the Arabidopsis transporter, *ZAT1*, which is thought to be involved in loading Zn into the vacuole (Assunção *et al*., 2001; Maser *et al*., 2001, Persans *et al*., 2001; van der Zaal, *et al*., 1999). *ZTP1* shares high homology to MTP1, a metal transporter from the Ni hyperaccumulator, *T. goesenginse*, which is a member of the CDF family of cation transporters. However *TgMTP1* localizes to the plasma membrane, while *TcZTP1* is thought to localize to the tonoplast, based on homology to *AtZAT1*.

Another metal transporter that has received significant attention in *T. caerulescens* is *TcHMA4*, which was first identified via yeast complementation and screening of transgenic yeast for increased Cd tolerance (Bernard *et al*., 2004, Papoyan and Kochian, 2004). *TcHMA4* is a member of the P-type ATPase superfamily, and more specifically, the P_{1B} subfamily of ATPases that are purported to transport heavy metals. *TcHMA4* was found to be expressed primarily in roots and its expression is induced by both Zn deficiency and high Zn treatments, as well as in response to high Cd (Papoyan and Kochian, 2004). The Arabidopsis homolog of *TcHMA4* has been characterized in detail and has been shown to be expressed primarily in the root stele and is believed to be involved in loading of Zn into the xylem for transport to the shoots (Hussain *et al*., 2004; Sinclair *et al*., 2007; Verret *et al*., 2004). Overexpression of *AtHMA4* also leads to increased accumulation of Zn and Cd in the shoots of the transgenic Arabidopsis plants, further suggesting *HMA4* plays a role in loading metals into the xylem (Verret *et al*., 2004). Also when both *AtHMA4* and its close relative, *AtHMA2* are both knocked out in Arabidopsis, reduced Zn accumulation in the shoot is seen (Hussain *et al*.,

2004). As efficient translocation of metals from the root to the shoot is a hallmark of metal hyperaccumulators, it has been suggested that TcHMA4 may play a critical role in heavy metal transport to the shoot during hyperaccumulation (Papoyan and Kochian, 2004). Support for this hypothesis comes from work from another metal hyperaccumulator, *Arabidopsis halleri*. In a QTL mapping study in a cross between *A. halleri* and *A. lyrata* (a related non-accumulator), a major Cd tolerance QTL co-located with *AhHMA4* (Courbot *et al*., 2007).

Molecular studies of potential metal-binding ligands. As discussed above, physiological and biochemical studies have failed to clearly identify metal binding ligands which would be expected to be associated with the large concentrations of Zn and Cd accumulated in the leaves of hyperacumulators such as *T. caerulescens*. These physiological/biochemical studies have focused on organic acids, amino acids and phytochelatins. However, a few molecular studies have provided some circumstantial evidence that other organic compounds may play a role as metal-binding ligands. In the yeast complementation studied described above that identified TcHMA4 as a protein that conferred Cd tolerance in yeast, members of another family of genes, the metallothionein (MT) family, were also found to confer Cd tolerance to yeast (Papoyan and Kochian, 2004). Metallothioneins are cysteine rich, low molecular weight, metal binding proteins that can form mercaptide bonds with various metals and have been implicated in metal homeostasis primarily in mammals (Cobbett and Goldsbrough, 2002). Metallothioneins have been found in a wide range of organisms crossing a number of kingdoms, with all of the plant MT's falling in one of two main subclasses. For these two main groups, classification is based on where the cysteine residues thought to be

involved in the binding of the various metal ions are located (Cobbett and Goldbrough, 2002).

The first member of the MT family that was studied in some detail in *T. caerulescens* was *TcMT3* (Roosens *et al*., 2004). *TcMT3* is more highly expressed in the shoot but seems to have a basal level of expression throughout the plant under a wide variety of conditions. Furthermore, *TcMT3* expression is induced by Cd exposure. However, with regard to function, questions have arisen regarding the possible role of TcMT3 in metal hyperaccumulation. Based on functional studies in yeast, it appears that TcMT3 confers much greater levels of tolerance to Cu than Cd, and did not confer a measureable increase in Zn tolerance. The increase in yeast Cu tolerance (in a Cu sensitive mutant background) was more strongly conferred by TcMT3 compared with AtMT3 (Roosens *et al*., 2004). The authors speculated that possibly TcMT3 plays a role in metal tolerance by allowing *T. caerulescens* to maintain normal Cu homeostasis under conditions where high levels of Cd and Zn occur in the cytoplasm. Roosens and coworkers (2005) also studied and characterized two other MT's in *T. caerulescens*, *TcMT1* and *TcMT2*, in comparison with their *Arabidopsis thaliana* homologs. Constitutive expression of *TcMT1* and *TcMT2* was considerably higher than expression of their homologs in Arabidopsis. However, interestingly, with regards to functional metal tolerance assays in yeast, although both TcMT1 and TcMT2 conferred increased tolerance to Cd, Zn and Cu, the Arabidopsis homologs were either able to confer an equivalent level of tolerance to these metals for AtMT2 (compared to TcMT2), or a greater degree of Cd and Zn tolerance (for AtMT1). Based on these findings, the authors again speculated that in *T.*

caerulescens, MT's might be playing a role in maintaining proper Cu nutrition/homeostasis in the face of Cd and Zn hyperaccumulation.

Several studies have focused attention on the role of the non-protein amino acid, nicotianamine, in metal tolerance in *T. caerulescens*. Nicotianamine (NA) has been shown to be a chelator of several micronutrient metals and has been suggested to be involved in the movement of micronutrients and heavy metals throughout the plant (Stephan and Scholz, 1993). In ecotypes of *T. caerulescens* that also hyperaccumulate Ni, Mari *et al.* (2006) found via yeast complementation for Ni tolerance that the gene encoding nicotianamine synthase, *TcNAS1*, conferred high levels of Ni tolerance when expressed in yeast. *TcNAS1* was found to be expressed only in the shoots and induced in as little as 6 hours after treatment of Ni. However, after this same 6 hr exposure to Ni, high levels of nicotianamine were found in the roots, and NA-Ni complexes were also found in the xylem sap. This led the authors to speculate that in response to Ni, NA is translocated to the roots where it chelates the absorbed Ni and facilitates its transport to the shoot. Further evidence for a role for *TcNAS1* in Ni hyperaccumulation came from studies where *TcNAS1* was over expressed in transgenic *A. thaliana* plants (Piannelli *et al.*, 2005). This resulted in a significant increase in both plant Ni tolerance and Ni accumulation in the shoot. These findings suggest that a number of transporters need to be involved in the movement of both free NA and the NA-metal complexes in the plant. One possible family of transporters for this role is the Yellow Stripe Like family, where the first member of this family was identified as the putative Fe-phytosiderophore uptake transporter in maize roots, while other members were hypothesized to be involved in transport of NA-metal complexes (Curie *et*

al., 2001). Three members of the YSL family have been characterized in *T. caerulescens*, named *TcYSL3*, *TcYSL5*, and *TcYSL7* based on sequence homology to Arabidopsis YSLs (Gendre *et al*., 2006). All three genes were shown to be more highly expressed in *T. caerulescens* than their Arabidopsis counterparts. Both TcYSL3 and TcYSL7 were found to be expressed in the root stele, associated with the xylem. Functional analysis in yeast demonstrated that TcYSL3 had the ability to transport both Ni-NA and Fe-NA complexes in yeast. Based on these findings, the authors speculate that TcYSL3 may be involved in long distance Ni translocation in *T. caerulescens*.

Molecular studies on ecotypic variation in Cd hyperaccumulation. As described above in the section on the physiology of hyperaccumulation, *T. caerulescens* ecotypes from the south of France such as the Ganges ecotype are more effective Cd hyperaccumulators than other ecotypes, with the Prayon ecotype often used as the comparison. Lombi *et al*., (2002) noted that when both Ganges and Prayon were made Fe deficient, an increase in Cd accumulation was seen. This property had been studied previously for non-accumulator species, and it was shown in those studies that the root Fe transporter, IRT1, whose expression is induced by Fe deficiency, also can function as a Cd transporter (Vert *et al*., 2002; Connolly *et al*., 2002; Cohen *et al*., 1998). In *T. caerulescens*, this Fe deficiency-induced increase in Cd accumulation was shown to be much greater in Ganges versus Prayon, and a role for *TcIRT1* was suggested (Lombi *et al*., 2002). More recently, comparison of *TcIRT1* in *T. caerulescens* with *AtIRT1* in Arabidopsis has shown that there are actually two versions of the *IRT1* gene in *T. caerulescens,* and both the Ganges and Prayon ecotypes harbor both a full-length and a truncated version of the gene in their genomes. The Ganges

ecotype only expresses the full-length version of *TcIRT1* while the Prayon ecotype only expresses the truncated version. Interestingly, both the full length and truncated versions of TcIRT1 from the two ecotypes of *T. caerulescens* do not effectively transport Cd, compared with AtIRT1. Thus the authors concluded that TcIRT1 is not the high affinity root Cd transporter identified from the previous physiological comparisons of root Cd influx in Ganges versus Prayon (Plaza *et al*., 2007). Hence the molecular and physiological basis for the increased Cd accumulation in ecotypes such as Prayon and Ganges is still a mystery.

Global analysis of the T. caerulescens transcriptome: As described above for specific genes in *T. caerulescens* such as *TcZNT1*, *TcHMA4* and *TcMTP1*, their expression is much higher than the expression of their homologs in related non-accumulator plant species. This has led to the speculation that this "hyperexpression" of specific metal related genes may play a role in metal hyperaccumulation (see, for example, Pence *et al*., 2000; Pence 2002). Subsequently, using microarray technology, global analysis of the *T. caerulescens* transcriptome has shown this to be a broad response involving many genes. Several studies have compared the transcriptome of *T. caerulescens* and a related non-accumulator using commercially available Arabidopsis gene chips. Hammond *et al.* (2006) conducted a shoot transcriptome comparison between *T. caerulescens* and *T. arvense* using the Affymetrix *Arabidopsis thaliana* GeneChip array. The authors painstakingly conducted the necessary proof of concept analysis to validate that cross species hybridization to a genome wide array of a model species was able to yield reproducible results. In this study, a number of genes previously mentioned such as *ZNT1 MTP1* and *HMA4* all showed much higher

expression in the hyperaccumulator. Additionally, a number of other genes encoding transporters from the ZIP, CDF, and HMA families were also hyperexpressed in *T. caerulescens.* Previously, a similar comparative transcriptome analysis had been conducted between a different Zn/Cd hyperaccumulator, *A. halleri*, and *A. thaliana* using the same gene chip array (Becher *et al*., 2004; Weber *et al*., 2004). That study showed that hyperexpression may be a general property of metal hyperaccumulators, as a large number of genes were also found to be more highly expressed in *A. halleri* compared with *A. thaliana*. Interestingly only 16 of the genes shown to be more highly expressed in *A. halleri* also exhibited elevated expression in *T. caerulescens*, suggesting this set of genes might be important for hyperaccumulation in both plant species.

A comparison of the root transcriptomes of *T. caerulescens* versus *A. thaliana* using the Agilent Arabidopsis 3 60-mer oligonucleotide microarray identified a number of previously identified and also novel genes showing elevated expression in *T. caerulescens* (van de Mortel *et al.*, 2006). Many of these genes have been previously been identified as playing a role either in metal transport or sequestration. However a number of other novel genes were also looked at. For example, 131 transcriptional regulators were identified exhibiting at least a five-fold increase in expression in *T. caerulescens* compared to Arabidopsis. Interestingly, the authors also identified a suite of hyperexpressed genes involved in lignin biosynthesis. This finding correlated with increased lignification/suberization of the endodermal cell layer and even an occasional observation of two endodermal cell layers in roots of *T. caerulescens*. Based on these observations, the authors suggested the more strongly developed endodermis in the hyperaccumulator may

function to minimize the back movement of metals accumulated in the stele during the trans-root processes resulting in metal loading into the xylem.

Future Research and Concluding Remarks:

While significant progress has been made in understanding the physiology of metal hyperaccumulation since Brooks *et al*. (1977) first coined the term, hyperaccumulator, there is still much more to be done to understand this fascinating process. Progress has been made in understanding some of the fundamental transport physiology, and some of the notable genes involved in metal uptake and transport, as well as in possible mechanisms of metal tolerance have been identified. With the strong evidence that *HMA4* and/or *HMA2* may play a key role in metal loading into the xylem, which appears to be important in hyperaccumulators as they very efficiently translocate most of their absorbed metal to the shoot, more work on this specific transport step is needed. Furthermore, it is still not clear whether common or separate mechanisms are employed for the hyperaccumulation of Zn, Cd and sometimes Ni in this plant species. The significant difference in Cd hyperaccumulation between ecotypes from different regions of the world needs to be exploited more fully as an experimental tool to better understand Cd hyperaccumulation.

Although a number of different metal transporters have been implicated in metal hyperaccumulation, the transport function and regulation at the transcriptional and post-transcriptional levels for most of these transporters is still quite poorly understood. As pointed out earlier, sequence similarity may not be the best predictor of transport function. The comparison between *TcZNT1* and *AtZIP4*, while closely related at the DNA and protein sequence

level, appear to transport different micronutrient/heavy metals and their expression is also influenced by the plant status for different metals. It is clear we need to better understand the relationship between transporter structure and function, as well as the regulation of these transporters.

One of the most striking features of metal hyperaccumulators is the hyperexpression of whole suites of both metal-related, and nonmetal-related genes. It appears that this hyperexpression is important to the hyperaccumulation phenotype. However, almost nothing is known about the molecular basis for hyperexpression. Is this trait controlled by a small group of unique trans-acting factors, or by trans factors acting in concert with promoter elements unique to hyperaccumulators?

A better understanding of metal hyperaccumulation in *T. caerulescens* will certainly benefit from the development of more and better genetic resources. To date, only a handful of QTL analysis studies have been conducted with relatively small mapping populations. This is certainly an area of research that requires much more development.

While a number of soil-based trials have been conducted under controlled conditions, the real application in the field still needs to be investigated further. A few studies recently have looked at the feasibility of using this plant species for the remediation of moderately Cd contaminated soils and have shown some promise (McGrath *et al*., 2006; Yanai *et al*., 2006). Also with the wide amount of natural variation in different populations of *T. caerulescens,* increased selection for traits of interest may help improve its phytoremediation capacity. However, to date, only remediation of Ni contaminated sites using hyperaccumulator species other than *T. caerulescens* has been shown to be economically viable (Chaney *et al*., 2005).

T. caerulescens is a fascinating and useful model system not only for studying extreme metal hyperaccumulation, but also for better understanding micronutrient homeostasis and nutrition. We have only started to understand the mechanisms underlying this unique plant trait and there certainly are interesting times ahead. More broadly, with the wide diversity of plants as well as ecological niches that allow for life to flourish under harsh conditions, understanding the molecular and physiological basis that allow these extremeophiles to function in these unique niches could provide considerable basic information that will be useful both for improving crop production on marginal and degraded soils, and also for developing plants well suited for environmental remediation.

The subsequent chapters in this dissertation summarize and analyze findings from research investigating how the transport of Zn and Cd from the soil to the shoot is altered in the heavy metal hyperaccumulator, *T. caerulescens*, in comparison with two closely related nonaccumulator plants, *Thlaspi arvense* and *Arabidopsis thaliana*. Additionally ecotypic variation in Cd hyperaccumulation within *T. caerulescens* was exploited to identify candidate metal hyperaccumulation genes. These two approaches were used to identify key proteins involved in Zn and Cd uptake, regulation and sequestration. One component of the research was the functional characterization of the micronutrient transporter, TcZNT1, which had previously been suggested to be the root Zn/Cd uptake transporter in *T. caerulescens*. TcZNT1 was characterized in comparison to its closest sequence-based homolog in Arabidopsis, AtZIP4. From this research it was found that TcZNT1 is most likely involved in root Zn uptake from the soil. Evidence is also presented for a new functional role for this transporter in long distance Zn transport based on

high levels of *TcZNT1* gene expression in the root and leaf vasculature. While it was found that AtZIP4 shares a number of similarities to TcZNT1 with regards to metal transport and tissue-specific expression, we identified a major difference in that AtZIP4 is localized to the chloroplast while TcZNT1 is a plasma membrane transporter. Hence AtZIP4 must play a role in chloroplast micronutrient homeostasis. A second component of the research focused on potential transcriptional regulators of *T. caerulescens* Zn transporter genes. Using yeast complementation assays, we identified intriguing candidates for this role based on the ability of *T. caerulescens* transcription factors to activate the expression of a yeast high affinity Zn uptake transporter. These proteins are members of the E2F family of transcription factors that had previously been thought to be involved solely in cell cycle regulation. Here it was shown that one of these TcE2Fs binds to a putative E2F element in the TcZNT1 promoter. TcZNT1 is closely related in sequence to the yeast Zn transporter gene that was activated by the TcE2F's. However, to date, we have not shown direct activation of *TcZNT1* expression by TcE2F2. The final component of this research project exploited natural ecotypic variation in Cd hyperaccumulation between two *T. caerulescens* ecotypes, and used a comparative transcriptomics approach between these ecotypes to identify a candidate transporter for Cd hyperaccumulation. This transporter, TcHMA3, is a member of the P-type ATPase family of metal transporters and was found to be a vacuolar Cd transporter expressed in both the roots and shoots of *T. caerulescens*. Overexpression of this vacuolar transporter in transgenic Arabidopsis led to increased Cd tolerance and increased root Cd accumulation.

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CHAPTER II

Characterization of the high affinity Zn transporter, TcZNT1, and its Arabidopsis homolog, AtZIP4, for their role in micronutrient hyperaccumulation and homoeostasis

ABSTRACT

Metal hyperaccumulating plant species are endemic to metalliferous soils and are able to tolerate and accumulate metals in their above ground tissues to very high concentrations. One such hyperaccumulator, *Thlaspi caerulescens,* has been widely studied for its remarkable ability to tolerate toxic levels of zinc (Zn), cadmium (Cd), and, sometimes, nickel (Ni) in the soil, and to accumulate these metals to very high levels in the shoots. *Thlaspi caerulescens* can amass as much as three percent Zn and one percent Cd on a dry weight basis in the plant shoots with no signs of toxicity. Previous research on how this unique plant species tolerates such high levels of toxic metals identified TcZNT1 as a high affinity Zn transporter and low affinity Cd transporter which is expressed at high levels in the roots of *T. caerulescens*. While initial evidence suggested that TcZNT1 might be involved in Zn/Cd uptake from the soil, the findings presented here indicate that TcZNT1 may also have other roles in the movement of Zn throughout the plant, based on its high level of expression in the root and shoot vasculature. We have further characterized TcZNT1 and its homolog in Arabidopsis, AtZIP4, and have found many similarities and a few important differences between these two related transporters. Both transporters mediate Zn transport, and their expression is induced by Zn and Cu deficiency, as well as Cd stress. The most dramatic difference is in their membrane localization. The plasma membrane

localization of TcZNT1 fits with its proposed roles in root Zn uptake and long distance Zn transport in the root and shoot. Surprisingly, it was found that AtZIP4 is localized to the chloroplast. Hence it must play a role in chloroplast micronutrient homeostasis. The findings presented here clearly indicate that while these two transporters are very similar with regards to sequence, their proposed role(s) in plant micronutrient homeostasis and hyperaccumulation are very different.

INTRODUCTION

Zinc (Zn) is an essential nutrient in both plants and animals and is required for a number of biological processes. Zn frequently plays a key role as a cofactor in enzymes such as RNA polymerase, alcohol dehydrogenase, and carbonic anhydrase, but Zn also plays a structural role in many proteins, including Zn finger motifs, and also in plasma membrane stability (Marshner, 1995). Zinc ions in the cell act as a structural bridge in many proteins, since this element does not readily undergo oxidation-reduction like iron (Fe) and copper (Cu) (Marshner, 1995). With such an essential role in so many aspects of the plant life cycle, a better understanding of Zn movement from soil to seed is necessary.

To date, only a handful of plant Zn transporters suggested to be involved in uptake have been identified from work in Arabidopsis. The first three, ZIP1, ZIP2 and ZIP3 were identified by Grotz et al., (1998) and then ZIP5 and 6 were identified later (Wu et al., 2009). These five transporters are members of a larger family of micronutrient transporters called the ZRT-IRT like Protein or ZIP family, which was named for its founding members in both yeast and *Arabidopsis*. Despite the identification of these transport proteins,

the route by which Zn enters and is transported throughout the plant is still poorly understood. The ZIP family of transporters has been suggested to be involved in transport of not only Zn, but also Fe, Zn, Cu, and Mn. With a possible role in the transport of a large number of essential micronutrients it is important to gain a better understanding of this metal transporter family. Also, due to its high degree of conservation in animals, fungi, plants and even bacteria, a better understanding of the function and role of ZIP family members in one species may help to elucidate micronutrient homeostasis in a number of organisms.

To date, the best understood ZIP transporters are those involved in yeast (*Saccharomyces cerevisiae*) Zn transport and homeostasis. In yeast, two ZIP transporters, ZRT1 and ZRT2, mediate high and low affinity Zn uptake into the cell, and a third, ZRT3, is involved in Zn transport out of the vacuole back into the cytosol (Zhao and Eide 1996a, 1996b; MacDiarmid et al, 2000). In plants, one member of the ZIP family, IRT1, is best known for its role in Fe uptake and homeostasis, but little is known about other transporters in the family and what role they may have in overall plant micronutrient homoeostasis (Eide et al., 1996; Vert et al., 2001, 2009; Eckhardt et al, 2001; Bughio et al., 2002; Plaza et al., 2007; Pedas et al., 2008). The ZIP family in plants is relatively large. In rice and Arabidopsis, there are 12 and 15 members, respectively, of which seven in both species have been characterized in at least a limited fashion (Grotz et al., 1998; Ramesh et al., 2003; Wintz et al., 2003; Ishimaru et al, 2005, Yang et al., 2007; Lin et al., 2009; Mäser et al., 2001; Wu et al., 2009). It appears that some of the plant ZIP transporters may be involved in Zn transport, with six Arabidopsi*s* and rice ZIPs suggested to be involved in Zn homeostasis, and one in *T. caerulescens*

(Grotz et al., 1998; Ramesh et al., 2003 Ishimaru et al, 2005, Yang et al., 2007; Lin et al., 2009; Eide et al., 1996; Korshunova et al., 1999; Vert et al, 2001; Pence et al., 2000; Wu et al., 2009).

The first metal transporter identified in hyperaccumulating plant species was TcZNT1 from the Zn/Cd heavy metal hyperacccumulator, *Thlaspi caerulescens* (Pence et al, 2000). When expressed in yeast, TcZNT1 was shown to mediate high affinity Zn transport as well as low affinity Cd uptake. TcZNT1 was suggested to be involved in uptake of Zn across the root-cell plasma membrane based on the similar uptake kinetics in Thlaspi roots and yeast expressing TcZNT1, and the correlation between higher *ZNT1* expression and root Zn uptake in a comparative study between *T. caerulescens* and the related non-accumulator, *T. arvense* (Lasat et al., 2000, Pence et al., 2000). Comparison of the TcZNT1 amino acid sequence with ZIP family members in Arabidopsis, which is relatively closely related to *T. caerulescens*, revealed that the closest sequence-based homolog is AtZIP4 in Arabidopsis, sharing almost 90 percent identity at the protein level. However, previous studies indicated that *AtZIP4* was unable to complement the ZHY3 yeast Zn uptake mutant, but may transport Cu based on the ability of AtZIP4 expression to complement the yeast Cu uptake mutant, *Δctr1* (Grotz et al., 1998, Wintz et al., 2003). *AtZIP4* has been shown to have increased transcript levels under both Zn and Cu deficiency in Arabidopsis, suggesting that it may also indirectly be involved in Zn nutrition (Wintz et al., 2003).

While the initial studies of TcZNT1 in *T. caerulescens* suggests it is a root Zn uptake transporter, a comprehensive characterization of this transporter has not been conducted to date. In this study, a detailed analysis of tissue and cell specific gene expression, protein localization, and transport

function in yeast and transgenic Arabidopsis was conducted for both TcZNT1 and its Arabidopsis homolog, AtZIP4, in order to better understand the role that each of these transporters play in micronutrient homeostasis.

RESULTS

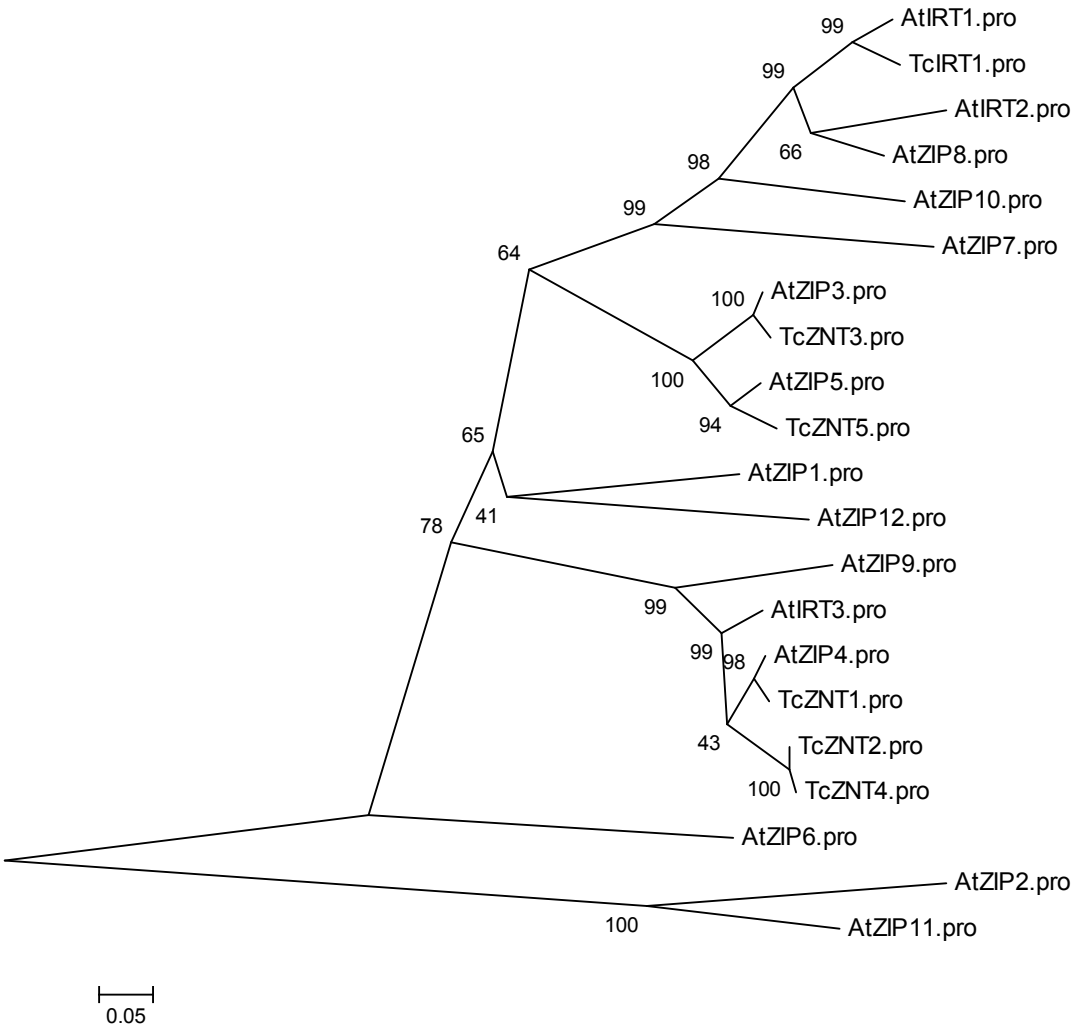
A comparison of the ZIP family members in *Arabidopsis thaliana* and *Thlaspi caerulescens* currently listed in NCBI reveals the existence of at least six different ZIP transporters in *Thlaspi* and 15 in Arabidopsis. As seen in Figure 2.1, TcZNT1 is most closely related based on sequence to AtZIP4, sharing 90.1 percent identity between the two proteins at the amino acid level. Each protein is predicted to contain 8 transmembrane domains with extracellular C and N termini. The most significantly different portion of the two proteins is between the 3rd and 4th transmembrane domains, and this region is thought to be intracellular in location and contains a histidine rich region which is believed to be involved in metal binding.

The sequence of *AtZIP4* isolated in this study has four nucleotide changes compared to the reference sequence listed in TAIR. Of these mutations two are silent. The other non-silent mutations involve a change from a T to a C, which alters the 32nd amino acid from serine to proline and a change from a T to an A located at the 295th amino acid which changes the amino acid from phenylalanine to isoleucine. Both of these changes are hydrophobic in nature.

Relative Expression of *TcZNT1* and *AtZIP4*

A comparative quantitative real time PCR analysis was undertaken on *TcZNT1* and *AtZIP4* to discern the response of the expression of these genes

Figure 2.1: Phylogenetic tree of the 15 Arabidopsis ZIP family members and the six *T. caerulescens* family members that are contained in NCBI. All of the *T. caerulescens* sequences listed are from the Prayon ecotype. Accession numbers for each gene listed are (AtZIP1: AAC24197, AtZIP2: AAC24198, AtZIP3: AAC24199, AtZIP4: AAB65480, AtZIP5: AAL38432, AtZIP6: AAL38433, AtZIP7: AAL38434, AtZIP8: AAL83293, AtZIP9: AAL38435, AtZIP10: AAL38436, AtZIP11: AAL67953, AtZIP12: AAL38437, AtIRT1: AAB01678, AtIRT2: NP_001031670, AtIRT3: NP_564766), TcZNT1: AF133267, TcZNT2: AF275752, TcZNT3, TcZNT4: AF292370, TcZNT5: AJ937738.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TcZNT5.pro
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to changes in plant micronutrient status (Figure 2.2). Under nutrient replete conditions, *TcZNT1* showed a 23-fold higher transcript level in the roots compared to *AtZIP4* when normalized to 18S levels from each species. Under low-Zn conditions, *TcZNT1* exhibited a two-fold increase in expression, while *AtZIP4* showed a 15-fold increase. However, *AtZIP4* expression in Zn deficient plants was still 4-fold less than *TcZNT1* expression in Zn deficient *T. caerulescens* plants. When plants were grown under low Cu conditions, root *AtZIP4* expression was increased 12-fold compared to roots on Cu replete plants, while *TcZNT1* showed about a 50% higher expression in response to low Cu. The last treatment assayed was Cd stress, where plants of both species were grown on nutrient solution containing 5 μM Cd. Imposition of Cd stress induced strong increases in both *TcZNT1* and *AtZIP4* transcript levels relative to 18S, with *TcZNT1* expression increasing about seven-fold compared with –Cd treated *T. caerulescens* seedlings, and *AtZIP4* expression was increased ten-fold. It should be noted that although Cd induced a greater increase in *AtZIP4* expression compared with its *Thlaspi* homolog, *TcZNT1* expression in *T. caerulescens* roots was still approximately eight times greater than *AtZIP4* expression in roots of Cd treated Arabidopsis seedlings.

Metal Transport by TcZNT1 and AtZIP4

To investigate the transport capabilities of both TcZNT1 and AtZIP4 proteins, each gene was cloned into the pFL61 yeast expression vector driven by the constitutive phosphoglycerate kinase promoter and this construct was then transformed into one of five different yeast backgrounds. The five different yeast lines consisted of four different metal uptake mutants defective in Zn (*Δzrt1/zrt2*), Cu (*Δctr1/ctr3*), Fe (*Δfet3/fet4*), or Mn (*Δsmf1*) uptake, as

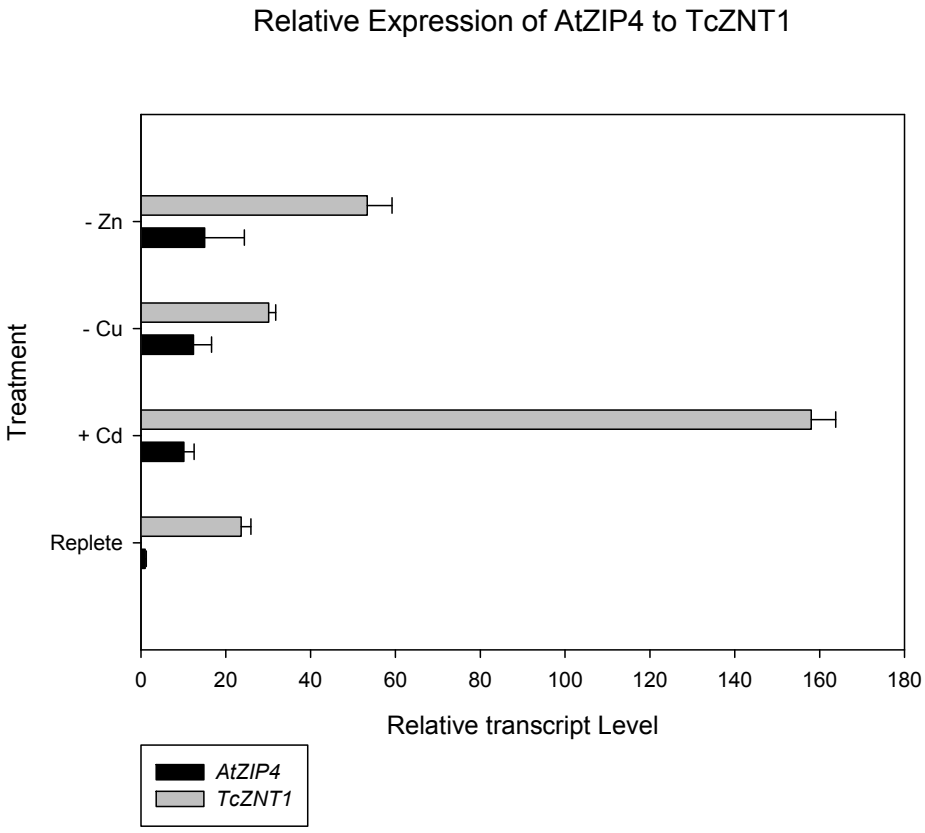


Figure 2.2: Quantitative real time PCR analysis of *TcZNT1* and *AtZIP4* expression in roots of *T. caerulescens* and *A. thaliana* plants grown on nutrient replete solution, and Zn-limiting, Cu-limiting, and +Cd nutrient solutions. The average transcript abundance from two biological replicates with SD is shown. Expression of shoots and roots were normalized to 18S levels for differences in expression among different treatments. *AtZIP4* root expression under replete nutrient conditions was set to one for comparison. Gray bars represent *TcZNT1* expression, and black bars represent *AtZIP4* expression.

well as wild type yeast which was used to study TcZNT1 and AtZIP4 Cd transport. Transport was assayed by the ability of the two transporters to restore growth on low Cu, Fe, Zn, or Mn in the appropriate mutant strain.

As shown in Figure 2.3, *TcZNT1* expression only complemented the *Δzrt1/zrt2* double mutant, indicating it could mediate Zn transport but not transport of Mn, Cu or Fe. Studies looking at which mutants were complemented by expression of *AtZIP4* showed that *AtZIP4* partially complemented the *Δzrt1/zrt2* Zn uptake mutant, however complementation of defective Cu transport in the *Δctr1/ctr3* double mutant could not be seen (Figure 2.3). *AtZIP4* was also unable to complement the *Δfet3/fet4* or *Δsmf1* mutants. The inability of *AtZIP4* to complement the *ctr1/ctr3* mutant, which had been shown in a previous study (Wintz et al, 2003), led us to mutate the two non-conservative amino acids changes in AtZIP4 cloned here back to the reference sequence listed by TAIR, to determine if these amino acid changes were involved in altering the selectivity of the transporter for either Cu or Zn. When these mutations were made and re-expressed in the four metal uptake mutant yeast backgrounds, no notable changes in complementation were seen compared to that of the initial version of AtZIP4, except for an apparent slight improvement in the ability to transport Zn (Figure 2.3).

Cd accumulation in wild type DY1457 cells expressing *TcZNT1* did not take up significantly more Cd than yeast expressing the empty vector (Figure 2.4A). However Cd accumulation by cells expressing *AtZIP4* was significantly greater than that of cells expressing either the empty vector or *TcZNT1*. *AtZIP4* or the reference *AtZIP4* expressing cells accumulated approximately 50% more Cd over the 1 hr uptake period (Figure 2.4A). When the same constructs were expressed in the *zrt1/zrt2* Zn uptake defective yeast mutant,

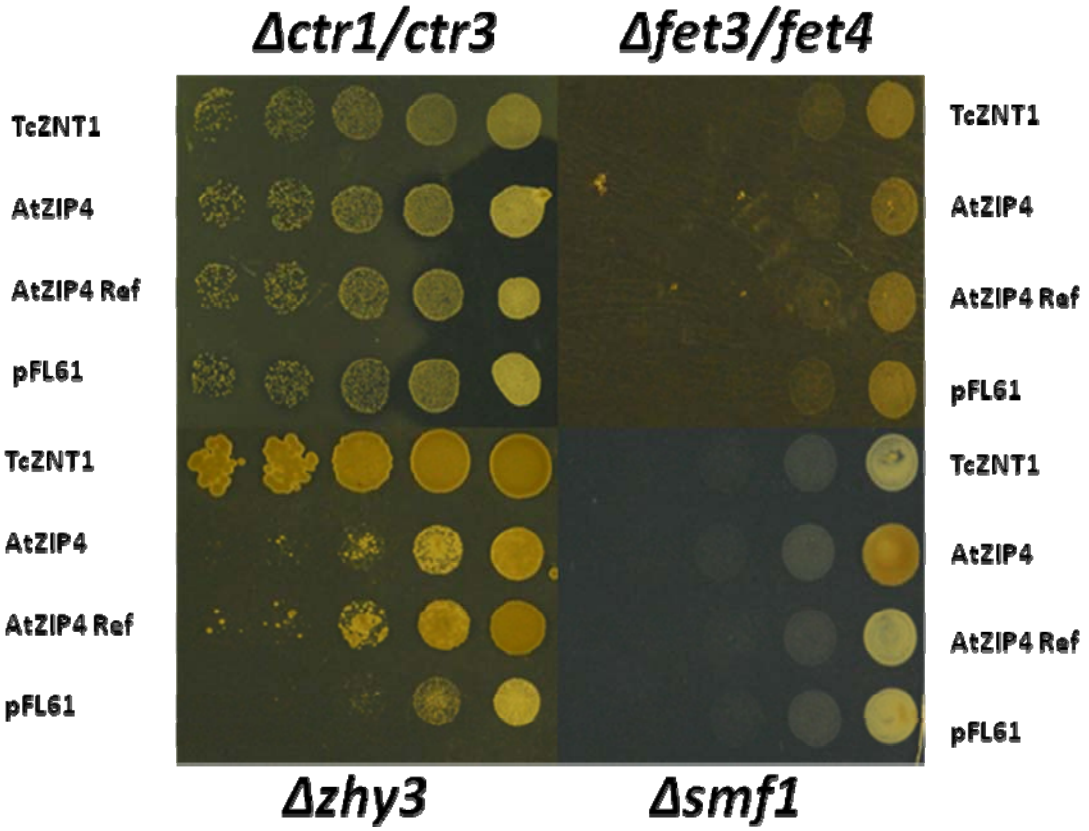


Figure 2.3: Complementation of yeast mutants defective in the uptake of Cu (Δ*ctr1/ctr3*), Fe (Δ*fet3/fet4*), Zn (Δ*zrt1/zrt2*), or Mn (Δ*smf1*), expressing either *TcZNT1*, *AtZIP4*, or the empty vector, pFL61. *TcZNT1* and *AtZIP4* were analyzed for their ability to transport Zn, Cu, Fe, or Mn via complementation of the specific mutant and growth on Cu, Fe, Zn, or Mn limiting media. From these complementation assays, TcZNT1 and AtZIP4 both appear to transport Zn. Complementation is shown after 72 hrs of growth on selective media containing the limiting micronutrient of interest. Each spot (from the right of each photograph) represents a 1:10 dilution of the culture starting with an O.D. of 1.

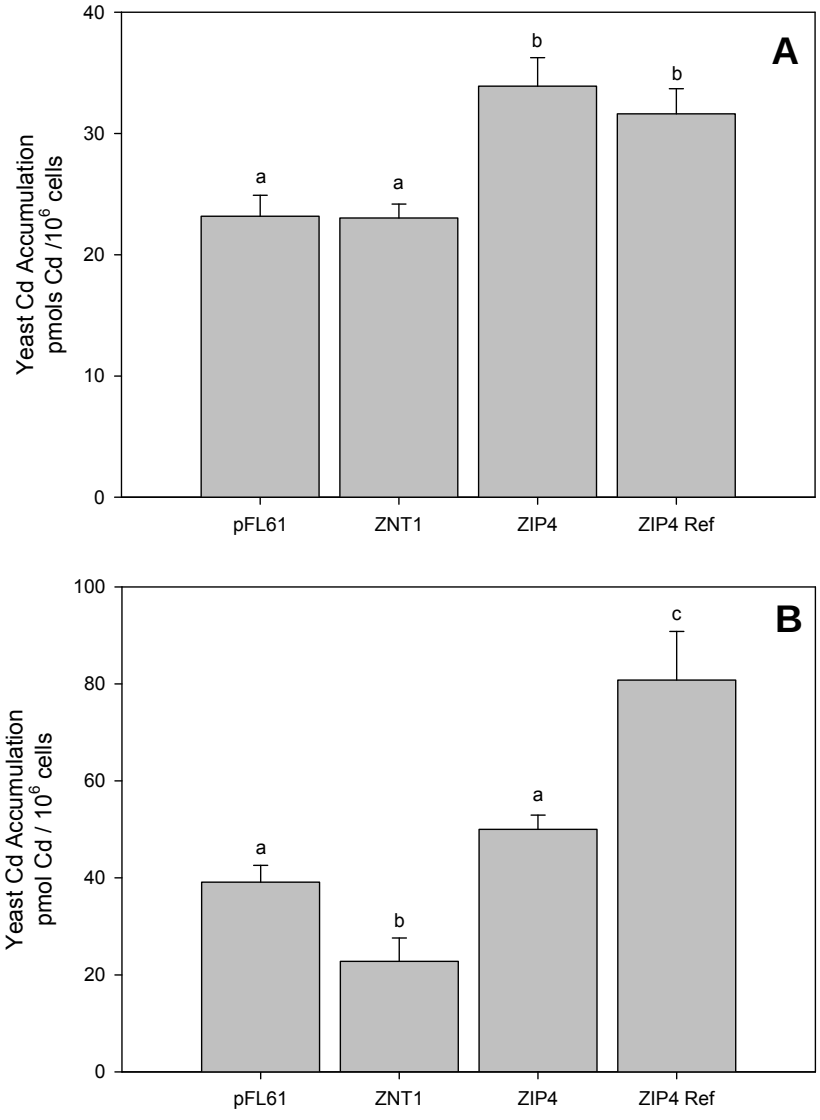
TcZNT1 expressing cells took up considerably less Cd than yeast expressing the empty vector or *AtZIP4*. ZHY3 cells expressing the reference version of *AtZIP4* accumulated significantly more Cd than did empty vector expressing cells (Figure 2.4B).

Tissue Specific *TcZNT1* and *AtZIP4* Localization using a Promoter::Reporter

A 1.1 kb region upstream of the start codon of *TcZNT1* was cloned in front of a YFP reporter and transformed into transgenic Col-0 plants. T_2 generation homozygous lines were recovered and gene expression was assayed. When the transgenic Arabidopsis seedlings were grown on media lacking Zn for 3 weeks, activation of the *TcZNT1* promoter::YFP reporter was found to be broadly distributed throughout cells of the root tip, including root cap, apical meristem, epidermis and stelar region (Figure 2.5A). It should be noted that earlier in the time course of Zn deficiency (2 weeks on –Zn media), *TcZNT1* expression was not seen in the root apex (data not shown). Further back in mature root regions, *TcZNT1* expression was found to be localized more predominantly to the stele, especially in the pericycle and stelar parenchyma cells, although expression is also seen in the root epidermis and cortex (Figure 2.5B-E). In the shoot, expression was seen in the vasculature and leaf guard cells, as was previously reported (data not shown) (Küpper et al, 2007).

A similar pattern for root *AtZIP4* expression was seen for transgenic Arabidopsis plants grown on media lacking Zn for two weeks transformed with a 1kb *AtZIP4* promoter::GUS reporter (Figure 2.5 F-I). Expression of the GUS reporter was highest in the root stele as well as in the leaf vasculature and

Figure 2.4: Cd accumulation in yeast expressing either TcZNT1, AtZIP4 or the AtZIP4 reference sequence, or the empty vector in: A) the wild type DY1457 background, B) the ZHY3 background. Cells in both A and B were grown on SC-URA to an OD of 1, then allowed to accumulate Cd from SC-URA media + 50 μM $CdSO_4$ for one hour, and then analyzed for Cd content via ICP-AES. Data are the means with SE (n=3). Significance was determined using ANOVA Tukey's post hoc analysis; letters indicate significance difference (p-val < 0.05)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guard cells (data not shown), but again as with *TcZNT1*, lower levels of expression were seen in the root epidermis and cortex.

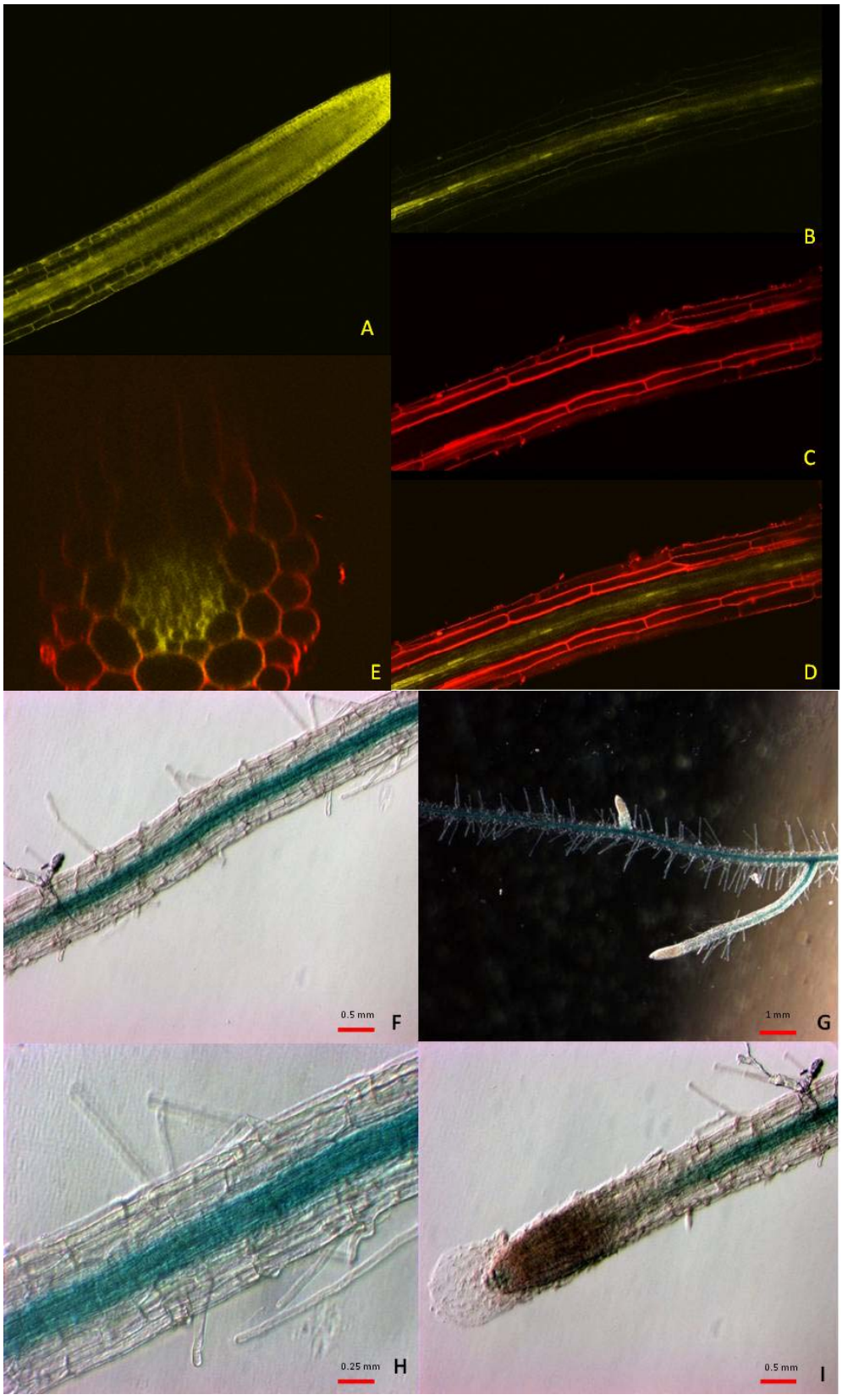
The influence of Cu deficiency on *TcZNT1* or *AtZIP4* expression was also examined, and similar to the qRT-PCR data in Figure 2.2, Cu deficiency had a greater stimulatory effect on *AtZIP4* promoter activity (data not shown).

To verify that the expression patterns seen in Arabidopsis were an accurate representation of what is seen in *Thlaspi* roots, a TcZNT1 peptide antibody was generated to a portion of the protein between transmembrane domains 3 and 4. As seen in Figure 2.6, immunolocalization of TcZNT1 in *Thlaspi* roots showed that the protein expression pattern was similar to the pattern of *TcZNT1* gene expression in transgenic Arabidopsis that was presented in Figure 2.5. The highest TcZNT1 protein expression was seen in the stele, but TcZNT1 protein was also seen in most cell types of the root including the epidermis and cortex. Furthermore, the TcZNT1 protein was localized to the cell periphery, consistent with a plasma membrane localization. TcZNT1 protein abundance also showed a strong Zn dependence, with the highest levels of protein seen in roots of –Zn grown Thlaspi plants. TcZNT1 protein abundance decreased as Zn concentration increased in the media (Figure 2.6A-C).

Protein Localization

Both the TcZNT1 and AtZIP4 proteins were fused to eGFP protein on either the N and C terminus and driven by a double 35S promoter with a translational enhancer and transiently expressed in Arabidopsis protoplasts. As seen in Figure 2.7, when protoplasts were imaged with a confocal

Figure 2.5: Tissue localization of *AtZIP4* and *TcZNT1* expression in transgenic Arabidopsis. Arabidopsis seedlings were transformed with the *TcZNT1* promoter (the DNA sequence spanning 1.1 kb upstream of the *TcZNT1* start codon) driving a YFP reporter, or the *AtZIP4* promoter (the DNA sequence spanning 1.1 kb upstream of the *AtZIP4* start codon) driving a GUS reporter. The seedlings were grown for either two or three weeks on a modified Johnson's solution without Zn. ***TcZNT1*p::YFP expression in:** A) root tip, B) root region 1 cm back from the root tip, C) root region 1 cm back from the root tip with cell walls stained with 1% propidium iodide, D) Overlay of the images from B and C, E) cross section of the root 1 cm back from the root tip which shows that *TcZNT1* is most highly expressed in the stele (pericycle, stellar parenchyma cells and protoxylem). ***AtZIP4*p::GUS expression in**: F) root tip, G) root region 1cm back from the root tip, H) lateral root 1.5 cm back from root tip, I) lower magnification image of root in H.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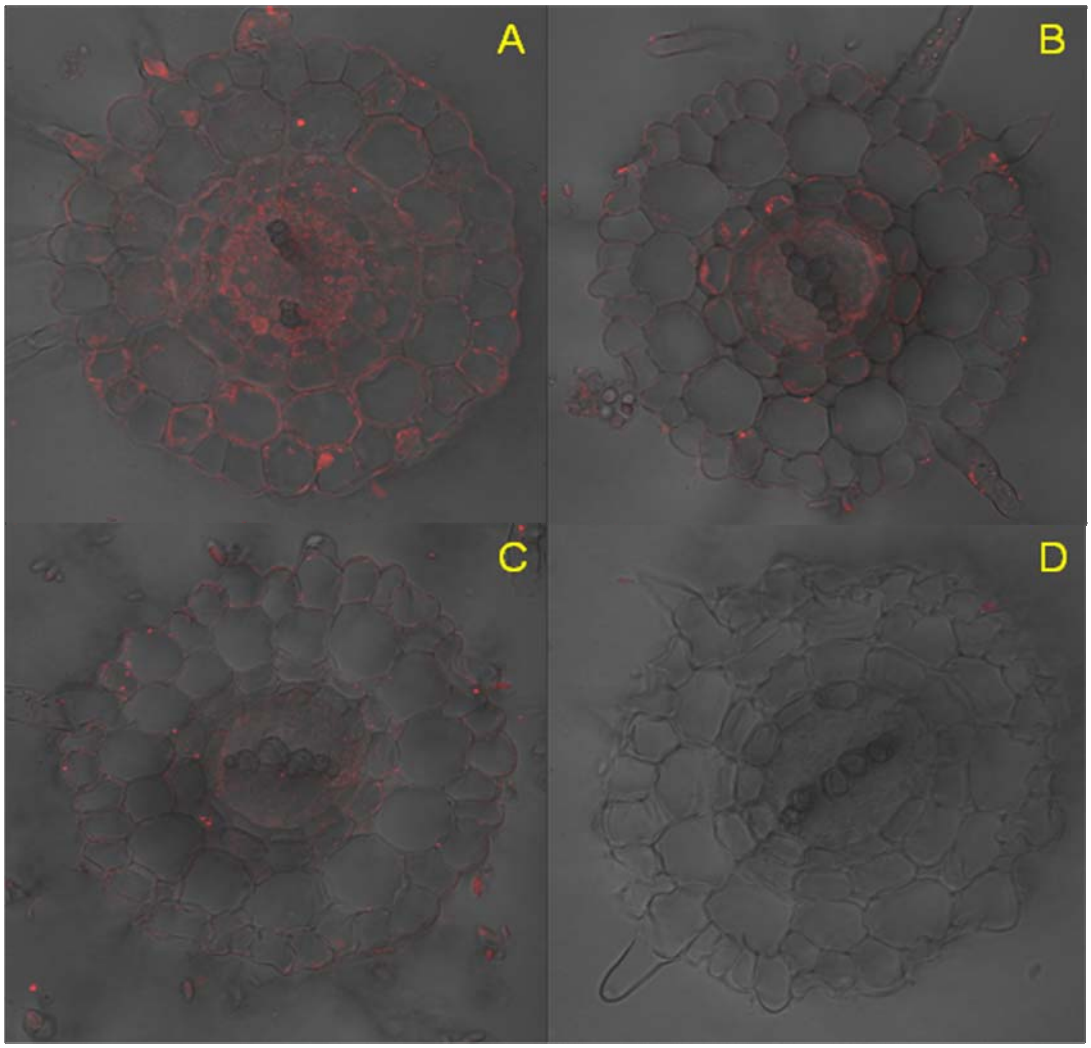


Figure 2.6: Root cross section showing immunolocalization of TcZNT1 antibody in *T. caerulescens* roots grown on: A) no Zn, B) 5 µM Zn, C) 100 µM Zn, D) no antibody for plants grown on no Zn. Figure provided by Dr. Jian Feng Ma.

microscope, eGFP:TcZNT1 was found to be localized to the plasma membrane, whereas eGFP:AtZIP4 fusion protein preliminary localized to the chloroplast. Because of the diffuse AtZIP4-eGFP fluorescence in the chloroplast, we will investigate the chloroplast localization for this protein in more detail in the future. It should be noted when the AtZIP4 amino acid sequence was analyzed using iPSORT, neither a mitochondrial nor a chloroplast targeting was indicated.

Over Expression of *TcZNT1* and *AtZIP4* in Transgenic Arabidopsis

The possible function of *TcZNT1* or *AtZIP4* in plant micronutrient nutrition was also examined in transgenic Arabidopsis seedlings overexpressing each gene under the control of the CmV35S promoter. When seedlings overexpressing *TcZNT1* were grown on high Zn levels in the nutrient solution (30μM Zn), plants showed a moderate but statistically significant increase in susceptibility to Zn toxicity compared to wild type Col-0 seedlings. Root growth in the three transgenic lines exhibited a 40-50 % inhibition of root growth by high Zn, where as root growth in Col-0 was inhibited 70% (Figure 2.8A). These same overexpression lines also accumulated up to 50% more Zn than that seen in wild type Col-0 alone (Figure 2.9A). When *AtZIP4* was over expressed there was a small increase in tolerance to high levels of Zn in the media, with the best performing three lines showing up to 30 percent greater root growth than Col-0 plants (Figure 2.8B). These same *AtZIP4* overexpression lines accumulated up to 30% less Zn than did wild type Col-0 plants (Figure 2.9B).

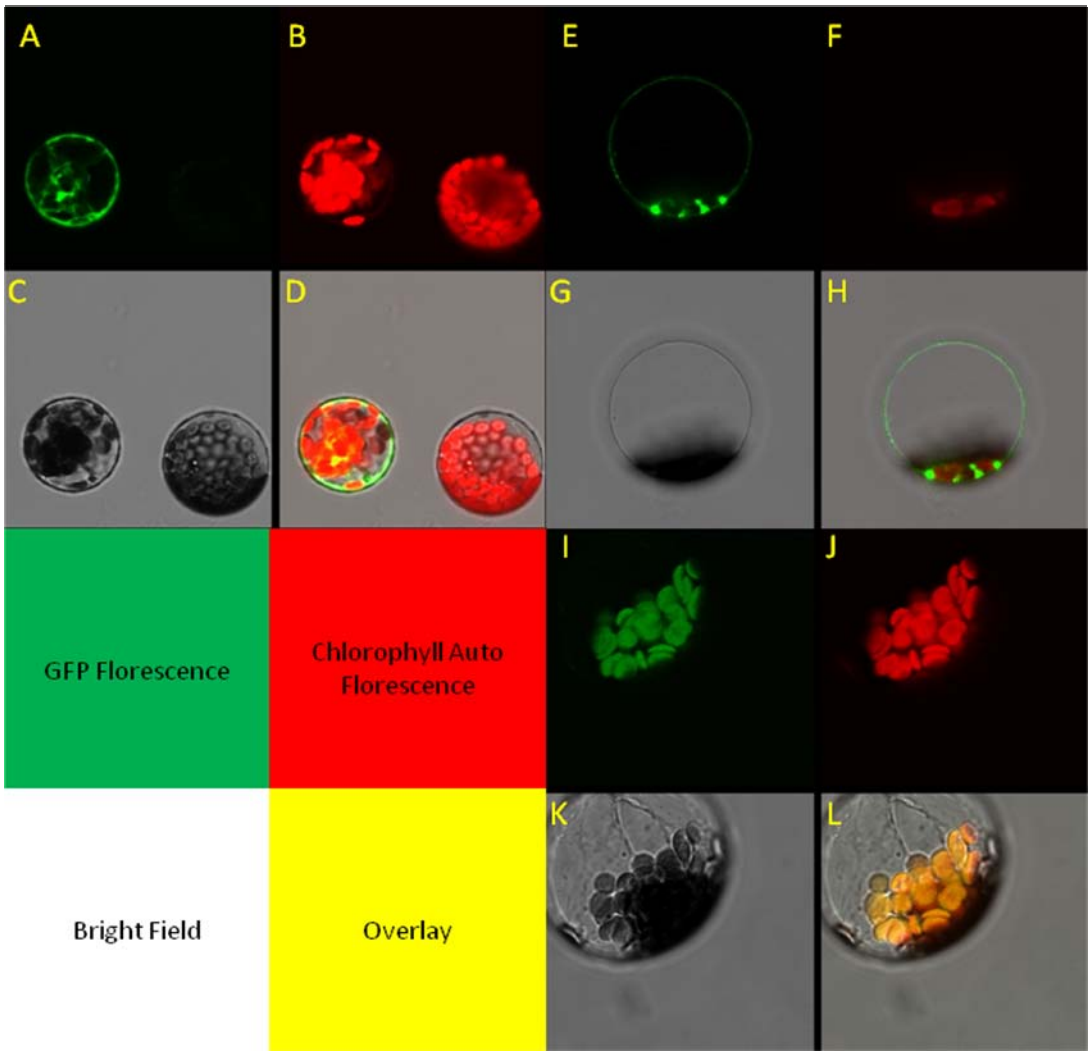
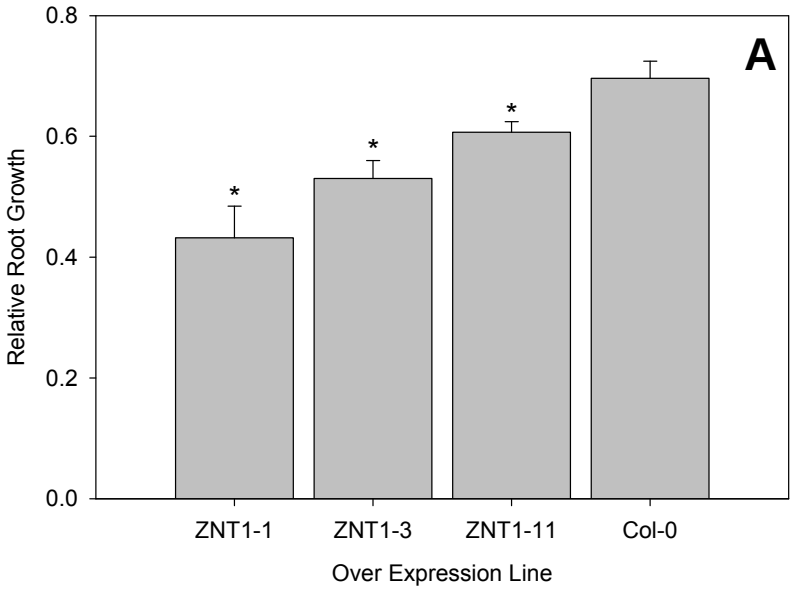


Figure 2.7: Sub-cellular localization of either TcZNT1 or AtZIP4 GFP transiently expressed in Arabidopsis protoplasts for 16 hours. Each box of four panels consists of the GFP image (upper left), chlorophyll fluorescence (upper right), bright field image (lower left), and a combined image of the other three channels (lower right) A-D) Cytosolic::eGFP construct, E-H) eGFP::TcZNT1, I-L) eGFP::AtZIP4

Figure 2.8: Over expression of *AtZIP4* and *TcZNT1* in transgenic Arabidopsis seedlings. The graph depicts Zn tolerance determined as relative root growth (root growth in high Zn media divided by control root growth) of plants expressing either *TcZNT1* or *AtZIP4* grown for 14 days on either modified Johnson's solution (control) or a modified Johnson's solution with 30 μM $ZnSO_4$ (high Zn). A) Relative root growth for homozygous transgenic lines expressing *TcZNT1* compared with wild type Arabidopsis. B) Relative root growth for homozygous transgenic lines expressing *AtZIP4*. Data are the means with SE (n=3). Significance was determined using ANOVA Tukey's post hoc analysis. Asterisk indicates significant difference relative to Col-0 (p. val < 0.05).


A
Relative Root Growth
0.0
0.2
0.4
0.6
0.8
*
*
*
ZNT1-1
ZNT1-3
ZNT1-11
Col-0
Over Expression Line

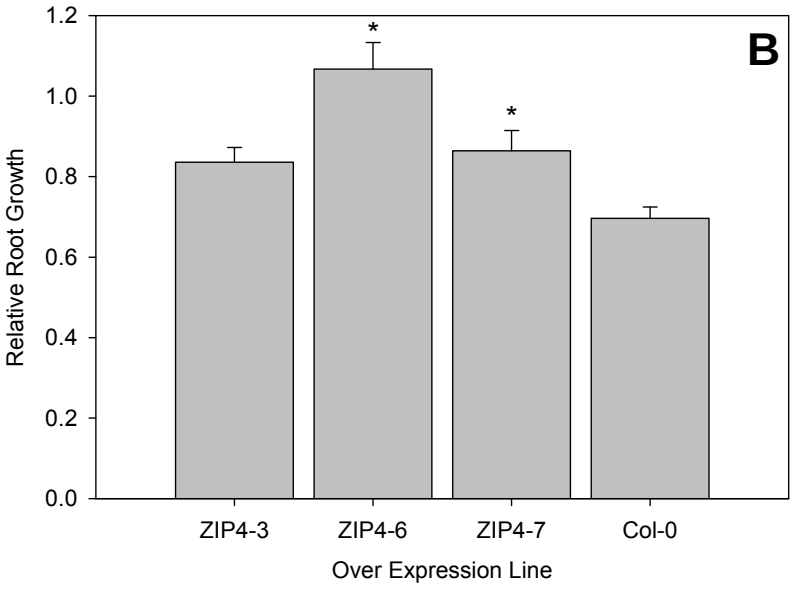
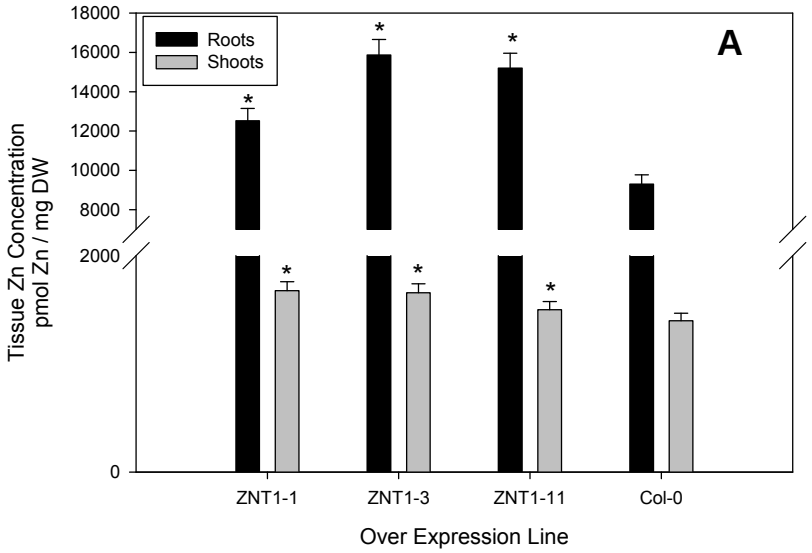
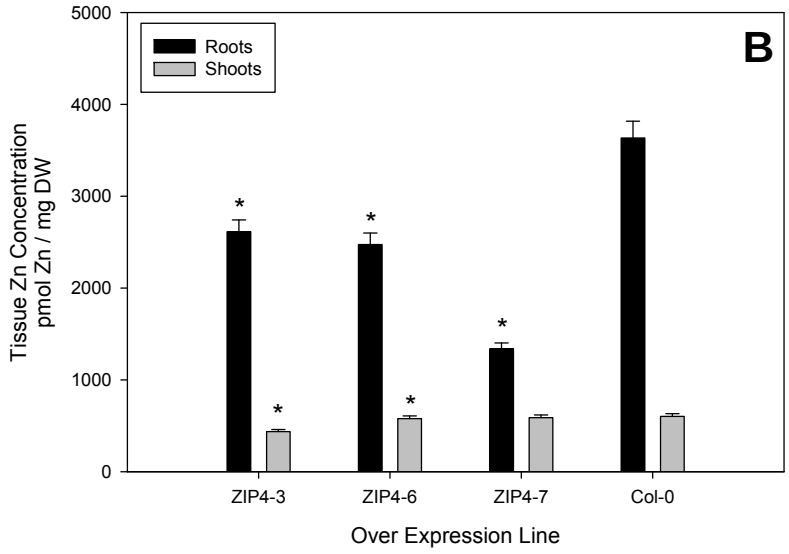

B
Relative Root Growth
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
*
ZIP4-3
ZIP4-6
ZIP4-7
Col-0
Over Expression Line

Figure 2.9: Zn concentrations in the roots and shoots of transgenic Arabidopsis seedlings overexpressing either *TcZNT1* or *AtZIP4*. Data are the means with SE (n=2). Tissue Zn concentrations were determined in plants grown for three weeks on complete nutrient solution with 5 μM Zn. Significance was determined using ANOVA Tukey's post hoc analysis. Asterisks indicate a significant difference vs Col-0 ($P < 0.05$)


A
Roots
Shoots
Tissue Zn Concentration
pmol Zn / mg DW
18000
16000
14000
12000
10000
8000
2000
0
ZNT1-1
ZNT1-3
ZNT1-11
Col-0
Over Expression Line


B
Roots
Shoots
Tissue Zn Concentration
pmol Zn / mg DW
5000
4000
3000
2000
1000
0
ZIP4-3
ZIP4-6
ZIP4-7
Col-0
Over Expression Line

DISCUSSION

The comparison of two closely related members of the ZIP family of proteins from two different *Brassica* species, TcZNT1 and AtZIP4, has revealed different possible functions for these two transporters despite their highly similar DNA and amino acid sequences. The two transporters did share several characteristics including the identity of the metal transported and tissues in which the genes were expressed. However, some of the differences in their transport and regulation suggest divergent roles for these two transporters. TcZNT1 was chosen for this study as it is very highly expressed in both roots and shoots of *T. caerulescens* and its Zn transport kinetics in yeast were similar to the kinetics of root Zn uptake in Thlaspi (Pence et al, 2000). AtZIP4 was chosen for this comparative study because it is the most similar ZIP transporter to TcZNT1 in *Arabidopsis thaliana*, which is a closely related non-accumulator plant species.

While both *TcZNT1* and *AtZIP4* were shown to mediate Zn transport in these studies, we were not able to confirm some previous findings for these transporters. These included Cd transport by *TcZNT1* and Cu transport by *AtZIP4*. Wintz et al (2003) previously was able to complement the copper transport defective yeast mutant *Δctr1* with AtZIP4, which restored yeast growth on low Cu. They did not show direct evidence for Cu transport via AtZIP4. We were not able to repeat this result using the *Δctr1/ctr3* Cu uptake mutant. Subsequently, we quantified Cu accumulation via ICP-MS analysis in the *Δctr1/ctr3* mutant expressing either AtZIP4 or the empty pFL61 vector and again found no evidence for Cu uptake via AtZIP4 (data not shown). In this study, the data presented in Figures 2.3 and 2.4 indicate that AtZIP4 can mediate Zn and Cd transport.

Also, in the current study, we demonstrated that TcZNT1 mediates Zn but not Cd uptake while in a previous publication from our lab, TcZNT1 expressed in yeast mediated high affinity Zn influx and low affinity Cd influx (Pence et al, 2000). There were two main differences between that previous study and the work performed here. For the research detailed in Pence et al (2000), a simple uptake solution consisting of $CaCl_2$ and MES was employed, while we used a full nutrient media supplemented with Cd for our yeast uptake study. The addition of other metals to the media in addition to just calcium may have interfered with Cd uptake here. This fits with the findings in Pence et al (2000) which indicated that TcZNT1 was a low affinity Cd transporter. The second difference is that in the Pence et al (2000) study, unidirectional Cd influx was quantified using radiotracer (^{109}Cd) flux techniques, while the methodology used here monitored net Cd accumulation. Thus it is possible that there is a significant Cd efflux in yeast cells, thus resulting in a much smaller net Cd uptake compared with the unidirectional Cd influx measured in Pence et al. (2000).

When comparing the relative transcript levels of *TcZNT1* and *AtZIP4*, both genes seem to respond to metal treatments in a similar fashion, showing increases in response to Zn and Cu deficiency, as well as Cd exposure, but the two genes show different relative levels of expression. Interestingly, the fold increases in relative transcript abundance were considerably greater for *AtZIP4* than for *TcZNT1*. This may be due to the fact that *TcZNT1* is expressed so highly to begin with in nutrient replete plants. As seen in Figure 2.2, both Zn and Cu deficiency increased transcript abundance for both genes, with Zn deficiency triggering a bigger increase in apparent gene expression.

These findings suggest the existence of at least a Zn responsive element in both promoters.

The most striking difference between the two genes is the fact that they localize to different membranes in the plant cell. While Grotz et al. (1998) suggested that AtZIP4 might localize to the chloroplast, no experimental evidence for subcellular localization of AtZIP4 has been presented in the literature until the current findings. Membrane prediction programs have been improved since Grotz et al. (1998) first suggested that AtZIP4 may localize to the chloroplast based on computational analysis, however iPSORT analysis of AtZIP4 in the current study did not identify a targeting sequence in the AtZIP4 protein that would suggest localization to the chloroplast. Here the AtZIP4-GFP localization data in Figure 2.7 suggests a tentative chloroplast localization for AtZIP4 while TcZNT1-GFP localization appears to be to the plasma membrane.

Further evidence in support of the GFP localization data comes from the Zn accumulation data for transgenic Arabidopsis expressing either *TcZNT1* or *AtZIP4.* The Zn accumulation phenotypes are consistent with localization to the plasma membrane. Plants expressing *TcZNT1* showed increased accumulation of Zn compared to Col-0 wild type seedlings or plants overexpressing *AtZIP4*, and this correlated with an increased sensitivity to high Zn in *TcZNT1* overexpressing plants. This fits a scenario where high levels of a root plasma membrane Zn transporter would result in enhanced Zn accumulation into the cytosol. The Zn accumulation phenotype for the Arabidopsis plants overexpressing *AtZIP4* was a bit surprising, as this resulted in a reduction in tissue Zn accumulation. These findings have led us to speculate that the role of the chloroplast in regulating micronutrient

homeostasis is more important than previously thought, and chloroplast micronutrient content may serve as a signal influencing micronutrient uptake into the cell.

The primary cells/tissues in which each gene is expressed are very similar. In the root, highest expression for both *TcZNT1* and *AtZIP4* is seen in the stele with lower expression in the epidermis and cortex (Figure 2.5). This expression pattern was verified at least for TcZNT1 protein, where immunolocalization of TcZNT1 protein in the *T. caerulescens* root was very similar to gene expression in transgenic Arabidopsis (Figure 2.6). In the shoot, both genes are most highly expressed in the vasculature and guard cells (data not shown). This tissue specific localization of expression is very similar to what was seen by Küpper et al (2007), who used quantitative in situ mRNA hybridization techniques to show that *TcZNT1* had its highest expression in the bundle sheath cells and guard cells. The very similar expression pattern for *TcZNT1* in Arabidopsis and TcZNT1 protein in *T. caerulescens* suggests that upstream activators of expression of these two transporter genes in response to Zn deficiency might recognize the same promoter element(s) in both *T. caerulescens* and *Arabidopsis thaliana* and that the Zn responsive elements may be conserved in plants, or at least within the *Brassicaceae*. The location of the TcZNT1 protein in *T. caerulescens* roots supports its role in Zn uptake from the soil, with expression seen in both the epidermis and the cortex of *Thlaspi* roots, as first suggested in Pence et al (2000). However the large level of expression seen in the stele suggests an additional role for TcZNT1 associated with long distance Zn transport. Possibly, as Zn moves radially across the root and crosses the endodermis, further radial movement within the stele could involve the sequential uptake

and then release of Zn as it moves to the xylem vessels for long distance transport.

While evidence presented here shows that AtZIP4 is not the functional homolog of TcZNT1, another member of the ZIP family, AtIRT3, might be a functional ortholog of TcZNT1, based on another recent study (Lin et la., 2009). Unlike *AtZIP4, AtIRT3* was shown to be plasma membrane localized, its expression is increased by Zn deficiency, and AtIRT3 can mediate Zn transport when expressed in yeast. Also, the expression pattern for *AtIRT3* is similar to that shown here for *TcZNT1* in roots and in Küpper et al. (2007) for the expression of *TcZNT1* in the leaves of *T. caerulescens*.

As for the role that each gene might play in the plant, it is likely that *TcZNT1* may be a major player in Zn uptake from the soil as previously suggested (Pence et al., 2000, Lasat et al., 2001). However, its localization to the stele suggests it also likely plays a major role in long distance transport of Zn to the shoot, which is a hallmark of metal hyperaccumulators and could account for such strong expression in the stele. The role of *AtZIP4* in plant micronutrient homeostasis is a little less clear. The previous reports that AtZIP4 could transport Cu would support its localization to the chloroplast, since the chloroplast seems to be strongly associated with copper homeostasis in plants. It may also be involved in bringing Zn into the chloroplast, as well helping to maintain the proper balance of micronutrients between the chloroplast and the cytosol.

MATERIALS AND METHODS

Cloning of *TcZNT1* and *AtZIP4*: *TcZNT1* was previously isolated for the work published in Pence et al., (2000) and this same clone was used in the

current studies. *AtZIP4* was cloned from total RNA isolated from *Arabidopsis thaliana* Col-0 seedlings grown for three weeks. For the first two weeks plants were grown on a modified Johnson's nutrient solution containing 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 µM KCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$, 0.1 µM $NiSO_4$, 1 µM $ZnSO_4$ and 0.5 µM $CuSO_4$, 2 mM MES pH 5.5. For the third week of growth the Johnson's solution was refreshed, but Zn was omitted from the solution. The primers used to isolate *AtZIP4* were ATGATCTTCGTCGATGTTCTTTGGAAATTG for the forward primer and CTAAGCCCAAATGGCGAGAGCAGACATAAG for the reverse primer. Corresponding clones were then cloned into the pGEM easy T vector (Promega Madison, WI USA) to transfer to pFL61 by digestion with *NotI*.

Yeast Studies: Yeast strains and nutritional requirements for the five different yeast strains used in these studies include, DY1457 (wildtype), *zrt1/zrt2* (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1*::LEU2 *zrt2*::HIS3), *fet3/fet4* (*MATa can1 his3 leu2 trp1 ura3 fet3*::HIS3 *fet4*::LEU2), *smf1* (SLY8; *MATa ura3 lys2 ade2 trp1 his3 leu2 smf1*::HIS3), and *ctr1/ctr3* (*MATa ctr1::ura3::Kanr ctr3::*TRP1 *his3 lys2-802* CUP1r). Each strain was maintained on YPD until introduction of either the gene of interest or empty vector. Cultures of each mutant containing one of the genes of interest were grown in liquid SC-URA to an optical density (O.D.) of 1 and then serially diluted 10, 100, 1000 and 10,000-fold. Each dilution was plated out onto the specific restrictive media for that mutant. For the *zrt1/zrt2* mutant, the restrictive media contained SC-URA plus 1mM EDTA and 500 µM $ZnCl_2$. For *smf1* the media was SC-URA containing 15 mM EGTA. The Δ*ctr1/ctr3* mutant was assayed for growth on

YPE media (10 grams yeast extract and 20 grams peptone per liter, and 10% ethanol). For the *fet3/fet4* mutant, cells were grown on SC-URA (pH 4.0) containing 80 μM bathophenanthroline disulfonate (BPS). For Cd accumulation studies, the cells were grown to an O.D. of 1 in SC-URA medium, then 50 μM $CdCl_2$ was added to the culture medium, and then the cells were allowed to accumulate Cd for one additional hour. Cells were then pelleted for five minutes at 3000g and washed twice with 5 mM $CaCl_2$. Cd accumulation was determined by ICP-AES.

Site Directed Mutagenesis: Site directed mutagenesis was carried out by designing a forward and reverse primer with the middle base pair mutated to change the desired amino acid paired with either the starting primer of the gene, or a primer located at the end of the gene. For the S32P mutation, the primers used were: ATCAGGAAGAGACTCTCTCTCAGAGT paired with CTAAGCCCAAATGGCGAGAGCAGACATAAG to amplify the five prime end of the gene and the primer, and ACTCTGAGAGAGAGTCTCTTCCTGAT paired with ATGATCTTCGTCGATGTTCTTTGGAAATTG to amplify the three prime end of the gene. Each PCR fragment was purified using a PCR column (QiaQuick, Qiagen, Valencia, CA) and mixed in equal proportions with the primers: ATGATCTTCGTCGATGTTCTTTGGAAATTG and CTAAGCCCAAATGGCGAGAGCAGACATAAG to obtain the full gene sequence containing the mutation. The PCR product was then TA cloned into pGEM easy T and sequenced to identify clones with the correct mutation. The same procedure was used for the F295I mutation using the S32P full length sequence as template and using the primer TCCTTCAAAGAACTGGTGAAATG and its reverse complement to incorporate the mutation into the S32P *ZIP4* sequence. The *ZIP4* reference

sequence was cloned into pGEM easy T and digested with *NotI* to clone into pFL61.

Protein Folding Prediction and Phylogenic Tree: The translated AtZIP4 and TcZNT1 proteins were run through the online site, WoLF pSORT (http://psort.ims.u-tokyo.ac.jp), to estimate the predicted membrane localization. In addition, the AtZIP4 protein was queried against SUBA (http://www.plantenergy.uwa.edu.au), to check predicted subcellular localization. To estimate the transmembrane domains, the translated proteins were run through Mobyle (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred). A comparison of the identified ZIP family members in *Thlaspi* and Arabidopsis was performed using Geneious (Version 4.84) (*www.geneious.com).*

Quantitative RT- PCR: For qRT-CR, plants were grown on a modified Johnson's solution containing 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 µM KCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$, 0.1 µM $NiSO_4$, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, and 2 mM MES (pH 5.5). For all metal treatments, plants were grown for two weeks on this media and then switched to one of the same composition except Zn or Cu was omitted or 5 µM $CdSO_4$ added, and plants were grown for an additional seven days. Plants were harvested, snap frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Total RNA was isolated using the Plant RNeasy RNA mini kit (Qiagen, Valencia, CA). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Transcript levels were measured using GoTaq® qPCR Master Mix (Promega, Madison, WI) with the primer pair ATCCTCTGTGATGCTGGCGAATC and CAGGGCTATGCGAGTTGAAAGA. Primers were designed to amplify a

homologous region of both *TcZNT1* and *AtZIP4*. A total of 25 ng of purified cDNA was used in each reaction to compare transcript levels across species. 18S was used as an internal reference and was amplified using the primer pair CGCTATTGGAGCTGGAATTACC and AATCCCTTAACGAGGATCCATTG to normalize across treatments and species. One ng of cDNA was used in these reactions since expression levels were high. Quantitative real-time RT-PCR was performed using an ABI 7500 real-time PCR system and SYBR Green kit (Applied Biosystems). PCR conditions used were 95 C for 5 min followed by 40 cycles of 95 C for 30 sec, 50 C for 30 sec and 60 C for 1 min. A dissociation curve was performed after each of the two biological replicates to ensure only one product was being amplified. *AtZIP4*, *TcZNT1* and 18S were amplified using normal PCR conditions to ensure amplification of the target gene and then cloned into pGEM easy T (Promega, Madison, WI) for target verification.

Protein Localization: *TcZNT1* and *AtZIP4* were both cloned into either pSAT6 or pSAT1 eGFPs, in frame on either the C or N terminus of the proteins, and transiently expressed in freshly isolated protoplasts from 3-4 week old Arabidopsis seedlings according to Sheen et al, (2001). Confocal images were taken 15-18 hours post transfection of the *Arabidopsis* protoplasts. Images were taken on a Leica TCS-SP5 confocal microscope (Leica Microsystems, Exton, PA USA) with a 63 x magnification, numerical aperture 1.2, water immersion objective. The eGFP was excited with the blue argon ion laser (488 nm), and the emitted fluorescence was collected from 505 nm to 545 nm.

Promoter Localization: A 1 kb section of *AtZIP4* was cloned upstream of the transcriptional start site into pBI101 and incorporated into *Arabidopsis thaliana*

ecotype Columbia via *Agrobacterium tumefaciens* line C58, using the floral dip method for stable transformation (Clough and Bent, 1998; modified from Bechtold et al. 1993). Plants were selected on half -strength MS with 50 µg/mL Kanamycin, and surviving seedlings were then transferred to soil and grown to seed. T_2 seeds were then assayed for GUS activity by growing them for seven to ten days on a modified Johnson's solution (listed above) containing no Zn. *AtZIP4*p:: GUS plants were then assayed for GUS activity in a solution containing 1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid in 50 mM Na_2HPO_4, pH 7.0 and 0.1% Triton X-100 for approximately four hours . The reaction was stopped by changing out the GUS solution and replacing it with 95% ethanol. For the *TcZNT1*p::YFP promoter, a 850 bp section upstream of the transcriptional start was isolating using genome walking, from the *T. caerulescens* genome and then cloned into pBAR upstream of a YFP reporter. The pBAR construct was transformed into *Arabidopsis thaliana* ecotype Colombia plants via *Agrobacterium* transformation. Transformants growing in soil were selected by spraying 1-2 week old plants with 150 µg/mL of Glufosinate every day for a week. To check for *TcZNT1* promoter activation, T_2 plants were grown for seven to 10 days in the same way as the *AtZIP4*p::GUS plants, but assayed for YFP expression using a Leica TCS-SP5 confocal microscope (Leica Microsystems, Exton, PA USA) with a 20X objective, numerical aperture 1.2, water immersion objective. To better define structures in the roots, the cell wall was stained using 0.1% propidium iodine.

Immunohistological Staining

Seedlings of *Thlaspi caerulescens* (ecotype Prayon) were grown in the same modified Johnsons solution listed above containing 5 µM Zn for 50 days and

then transferred to solutions with 0, 5, or 100 μM Zn, respectively. After four days of growth in these solutions, the roots were excised and used for immunohistological staining. Antibodies against TcZNT1 were obtained by immunizing rabbits with the synthetic peptide C-HGQSHGHVHVHGSHDVENG (positions 201 to 219 of TcZNT1). The procedures for immunostaining were followed as described previously (Yamaji and Ma, 2007). Fluorescence was observed using a laser-scanning confocal microscope (LSM700; Carl Zeiss).

Over expression of *TcZNT1* and *AtZIP4* in planta: *AtZIP4* was cloned into pBAR and *TcZNT1* was cloned into the pBI101 vector downstream of the 35S promoter and then transformed into *Arabidopsis thaliana* Col-0 via agrobacterium-mediated transformation using the C58 line. Soil-grown transformants were selected with 150 μg/mL of BASTA or grown out on half-strength MS with 50 mg/mL Kanamycin until true breeding lines could be obtained. Seeds of *AtZIP4* or *TcZNT1* overexpression lines were surface sterilized in dilute bleach (0.5% NaOCl) and then with 50% ethanol, washed five times with ultra pure water before being imbibed in 0.1% (w/v) low melting point agarose at 4^{o} C for five days. Homozygous lines were then grown on sufficient (1 μM $ZnSO_4$) or high Zn (30 μM $ZnSO_4$) media for two weeks and pictures of the roots were taken using a Nikon D200 camera with a 60 mm lens. Total root length of each plant was determined using Root Reader 2D software (www.plantmineralnutrition.net). Relative root length was the calculated by dividing the mean total root length of each high Zn-treated plant by the mean total root length of the control (Zn sufficient) plants. The data were compared using a one way ANOVA with Tukey's test for post hoc analysis. Each line was grown three separate times and compared to wild

type Columbia for sensitivity to Zn. To study plant mineral content, true breeding overexpression lines were grown for three weeks on a modified Johnson's solution containing 5 μM $ZnSO_4$. Roots were desorbed for 15 mins in 5 mM $CaCl_2$ and plants were then separated into roots and shoots for mineral content analysis. Elemental analysis was carried out using ICP atomic absorption emission spectroscopy (ICP-AES; to determine root and shoot mineral content.

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CHAPTER III

E2F Transcription Factors and their Role in Plant Micronutrient Homeostasis. Do They Regulate Plant Zn Nutrition in Addition to the Cell Cycle?

ABSTRACT

Increasing our understanding regarding the mechanisms controlling heavy metal and micronutrient accumulation in plants will provide valuable insights into the control of nutrient flow from soil to shoot and then seed. An interesting model for studies into Zn transport and regulation of Zn homoeostasis is the Zn/Cd heavy metal hyperaccumulating plant species, *Thlaspi caerulescens,* which was used in the present studies to investigate Zn homeostasis in a plant species that can tolerate very high levels of Zn and Cd in the shoots. To begin to study plant Zn sensing and regulation of Zn homeostasis in *T. caerulescens*, a cDNA library was screened by complementation of a yeast mutant lacking the yeast transcription factor, ZAP1, which controls the transcriptional activation of both the high and low affinity yeast Zn uptake transporters, ZRT1 and ZRT2. Two transcription factors from the *T. caerulescens* E2F family, a family previously thought to be involved solely in cell cycle regulation, were found to activate the expression of *ZRT1* and to restore growth of the yeast *Δzap1* mutant on low Zn media. Changes in *TcE2F2* expression correlated with changes in plant Zn status and expression of the putative high affinity Zn uptake transporter, *TcZNT1*, suggesting a role for TcE2F2 in regulating Zn uptake and transport. Furthermore, *in vitro* studies demonstrated that TcE2F2 binds with high affinity to a consensus E2F-binding site found in the *TcZNT1* promoter. To date, the

focus on research on plant E2Fs has been for their well known role in cell cycle regulation. The findings presented here provide intriguing initial evidence for a novel role for E2F transcription factors in plant micronutrient homeostasis.

INTRODUCTION

Thlaspi caerulescens (J&C Presl) is a Zn/Cd hyperaccumulator which tolerates high exposures to Zn and Cd. Both metals accumulate to high levels in the shoot and seed when plants are grown on elevated levels of Zn or Cd in soil or nutrient solution (Brown et al., 1995a; Brown et al., 1995b; Chaney, 1993, Reeves and Brooks, 1983). This ability to hyperaccumulate heavy metals has intrigued plant biologists for many years for the possible value *T. caerulescens* may have in phytoremediation of Zn- and Cd-contaminated soils (Baker et al., 1994; Brown et al., 1995b). However, it's slow growth and diminished shoot biomass limits its usefulness (Ebbs et al., 1997). Exploiting the genetic potential of this species by transferring metal hyper accumulating traits to plants with a higher biomass may be an effective approach to generate new and novel metal accumulator plants (Brown et al., 1995a). To accomplish this goal, a better understanding of the basic molecular and physiological mechanisms responsible for Zn/Cd hyperaccumulation is needed. Since Zn hyperaccumulation in *T. caerulescens* is related to the elevated or altered expression of a number of Zn responsive genes, this plant may serve as a useful tool for studying plant mechanisms of Zn sensing and homeostasis (Pence et al, 2000; Lasat et al, 2000).

Comparison of Zn transport in *T. caerulescens* and the related non-accumulator species, *T. arvense*, have demonstrated that Zn transport is

altered at several sites along the transport path from the soil to the shoot. For example, root Zn influx is considerably greater in the hyperaccumulator species under a wide range of external Zn concentrations (Lasat et al., 1996). Furthermore, a significant fraction of the Zn transported into the root symplasm remains in the cytoplasm in *T. caerulescens*, whereas in *T. arvense* a larger fraction of the absorbed Zn is sequestered in the root vacuole and made unavailable for translocation to the shoot (Lasat et al., 1998). Associated with these two transport alterations, there is a much larger xylem loading of Zn (5 fold greater) in *T. caerulescens* than in *T. arvense* (Lasat et al., 1998). Finally, radiotracer (^{65}Zn) uptake experiments conducted with leaf sections and leaf protoplasts from *T. arvense* and *T. caerulescens* demonstrated a greater capacity in *T. caerulescens* to absorb Zn into leaf cells than in the non-accumulator *Thlaspi* (Lasat et al., 1998). Collectively, these results show that alterations of Zn transport in both roots and shoots contribute to Zn hyperaccumulation in *T. caerulescens*. What accounts for these transport differences remains unresolved and serves as a primary focus of the current investigations.

Heavy metal hyperaccumulation in *T. caerulescens* is linked to the increased expression of a number of genes encoding metal transporters in *T. caerulescens* compared to the related non-accumulators, *T. arvense* and *Arabidopsis thaliana*. These hyperexpressed metal (Zn) transporters appear to be involved in different aspects of Zn uptake, transport and homeostasis and include ZNT1, a putative plasma membrane-localized root Zn uptake transporter (Pence et al., 2000), the vacuolar metal transporter ZTP1/MTP1 (Assunçao et al., 2001), and the transporter involved in xylem metal loading, HMA4 (Hussein et al., 2004; Papoyan and Kochian, 2004, Bernard et al.

2004). The genes encoding these three transporters have all been shown to exhibit much higher expression in the hyperaccumulating plant species (Assunção et al., 2001; Bernard et al.; 2004, Papoyan and Kochian, 2004; Pence et al., 2000). This hyperexpression phenotype is not solely a trait *T. caerulescens* possesses, as the Cd hyperaccumulator, *Arabidopsis halleri*, also exhibits hyperexpression of a number of different genes involved in micronutrient and heavy metal Zn transport/homeostasis (Becher et al, 2004; Weber et al 2004, Talke et al., 2006). Little is currently known about what factors contribute to the hyperexpression of this suite of genes in the hyperaccumulating plant species and serves as additional justification for the current investigations. Recently, genome copy number has been suggested as a contributor to elevated expression of certain genes in *A. halleri* (Talke et al., 2006; Hanikenne et al., 2008).

While little is known about the regulation of plant genes involved in transporting Zn from the soil to the shoot and seed, the yeast model organism, *Saccharomyces cerevisiae*, has been well characterized in terms of the molecular basis of Zn homeostasis. In yeast, the *ZAP1* transcription factor has been found to control the expression of the high and low affinity Zn transporters, *ZRT1* and *ZRT2* (Zhao and Eide, 1997). A *ZAP1* loss of function mutant was shown to be unable to activate expression of *ZRT1* and *ZRT2*, limiting yeast Zn uptake and growth under low Zn conditions (Zhao and Eide, 1997). In the current study, we used a yeast functional complementation screen with a *T. caerulescens* a cDNA yeast expression library to identify transcription factors that are candidates for regulating plant Zn transporter expression.

RESULTS

Identification of candidate transcriptional regulators via yeast complementation

A yeast complementation strategy was used to identify *T. caerulescens* genes capable of regulating the expression of yeast genes encoding micronutrient/heavy metal transporters. This involved the transformation with a *T. caerulescens* cDNA library of the yeast mutant, *zap1*∆. This mutant lacks the ZAP1 transcription factor involved in regulation of expression of the yeast high affinity and low affinity Zn uptake transporters, ZRT1 and ZRT2, which are members of the ZIP family and are closely related to the *Arabidopsis* and *Thlaspi* Zn transporters, AtZIP4 and TcZNT1. Four hundred fifty thousand yeast transformants were screened for *Thlapsi* cDNA's that restored the ability of the yeast to grow on low Zn. This complementation screen identified three *T. caerulescens* genes that conferred the ability for the ∆*zap1* to grow normally under Zn limiting conditions. One of these genes was *TcZNT1*, which we previously identified as a high affinity Zn transporter localized to the plasma membrane and highly expressed in roots and shoots of *T. caerulescens* (Pence et al., 2000). In the case of *TcZNT1*, the gene encoding the *Thlaspi* Zn transporter driven by a constitutive promoter is simply replacing the function of the yeast ZRT1 and ZRT2 transporters in transporting Zn into the yeast cell.

The other two cDNA's that were able to complement the *zap1*∆ mutant are members of the E2F family of transcriptional regulators, which we have named *TcE2F1* and *TcE2F2* for their homology to *AtE2F1* and *AtE2F2*, respectively. On low Zn media, *TcE2F1* and *TcE2F2* were able to restore growth of the yeast mutant to levels near those of wild type yeast (Figure 3.1).

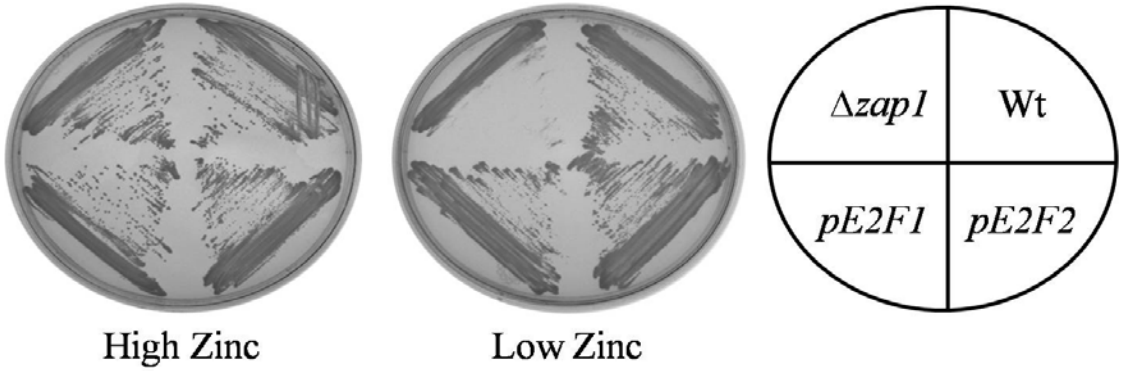


Figure 3.1: TcE2F's complement *zap1*Δ yeast mutant**.** Expression of *TcE2F1* and *TcE2F2* in the Δ*zap1* yeast mutant restored yeast growth on low Zn synthetic dextrose minimal media to a level of growth exhibited by the parental wild type (Wt). SD media was supplemented with a zinc chelate made from 1 mM EDTA and 2 mM $ZnSO_4$ (High Zn) or 500 □M $ZnSO_4$ (Low Zn). *TcE2F1* and *TcE2F2* were expressed behind the yeast phosphoglycerate kinase promoter in the pFL61 vector.

The predicted open reading frames for *TcE2F1* and *TcE2F2* are 444 and 387 amino acids in length and share greater than 85% homology to AtE2F1 and AtE2F2. Alignment of the TcE2F1 and TcE2F2 amino acid sequences with other known plant E2F proteins shows that the TcE2F proteins contain many of the same domains that help to define the E2F family. Both TcE2F1 and TcE2F2 have a putative nuclear localization signal, a leucine zipper domain which helps in dimerization with other proteins, a marked box domain which is important in protein-protein interactions, a DNA binding domain and finally a domain for interactions with a retinoblastoma (Rb) protein that is involved in regulation of the E2F family of proteins. All of these

domains in the Thlaspi E2Fs were found to be conserved with many of the Arabidopsis and mammalian E2F's identified to date. TcE2F2 exhibits a high homology to E2F's across many plant species, including Arabidopsis, rice and carrot (Figure 3.2). A comparison between the two *T. caerulescens* E2F proteins also reveals that the TcE2F1 and TcE2F2 proteins only share 40% identity, which may suggest they play different roles in the plant. Analysis of the proteins via PSORT predicts that both TcE2F1 and TcE2F2 will localize to the nucleus (Horton et al, 2006).

TcE2F1 and *TcE2F2* Gene Expression in Relation to Plant Zn Status

As seen in Figure 3.3, analysis of the relative transcript abundance for the *E2F* genes in roots and shoots of both *Thlaspi* species for plants grown under sufficient and high levels of Zn using semi-quantitative RT shows that both *TcE2F1* and *TcE2F2* are expressed at much higher levels in both roots and shoots of *T. caerulescens* compared to their counterparts in *T. arvense* in plants grown on both sufficient and high Zn levels (Fig 3.3A). Expression of both *E2F* genes in *T. caerulescens* and *T. arvense* responded to changes in plant Zn status, with *E2F1* being more responsive to Zn than *E2F2*. Furthermore, in roots of the non-accumulator species, *T. arvense*, both *E2F* genes appear to be induced by growth on sufficient levels of Zn (1 μM), compared to growth on high Zn (10 μM), while in *T. caerulescens* there was no change in *E2F* expression even in plants grown on Zn levels as high as 50 μM. Further analysis of expression of *TcE2F* and *TcE2F2* expression in *T. caerulescens* plants grown on a wider range of Zn levels (0 – 500 μM Zn), showed that there are different expression responses for the two E2F's in roots and shoots of *T. caerulescens* (Fig. 3.3B). In *T. caerulescens* roots, both

E2F genes exhibited their highest expression in Zn deficient and low Zn-grown plants, and expression decreased in plants grown on higher Zn levels (50 and 500 µM Zn). However in shoots, both *E2F*s showed steady and relatively low levels of expression in plants grown on 0 to 50 µM Zn, but in high Zn-grown plants (500 µM), there was a divergence in expression, with *TcE2F1* exhibiting a sharp increase in expression and *TcE2F2* showing no change in expression in relation to plants grown on the lower levels of Zn.

Possible Role of E2F Transcription Factors in Regulation of Zn Transporter Gene Expression

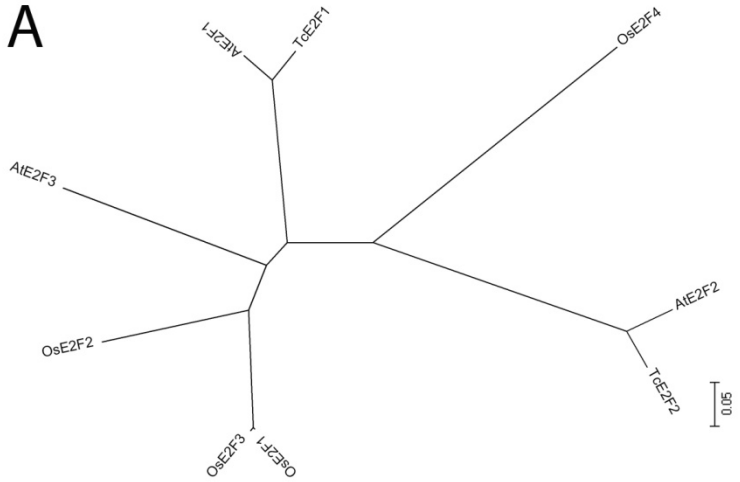
As previously mentioned, both *TcE2F1* and *TcE2F2* restored the ability of the yeast *zap1*∆ mutant to grow under low Zn conditions (Figure 3.1). To understand how the E2F proteins were able to restore yeast growth on low Zn media, expression of the high affinity Zn uptake transporter, *ZRT1*, was monitored in yeast grown on low and sufficient Zn in the yeast *zap1*∆ mutant. Activation of *ZRT1* expression was seen under both low Zn and replete Zn growth conditions in *zap1*∆ strains expressing either TcE2F protein, while no *ZRT1* expression was seen in the *zap1*∆ strain not expressing either E2F protein. Under these same growth conditions, wild type yeast show activation of *ZRT1* expression only under low Zn growth conditions (Fig. 3.4). No RNA could be collected from the *zap1*∆ yeast mutant grown under low Zn conditions since the mutant is unable to grow. Subsequent transformation of the E2Fs into a second yeast mutant, *zrt1/zrt2*∆, that does not grow on low Zn because it lacks both high and low affinity yeast Zn uptake transporters, was unable to restore growth (data not shown). These findings suggest that the *T. caerulescens* E2F1 and E2F2 transcription factors restored yeast growth via

activation of expression of the gene encoding the yeast high affinity transporter, ZRT1, which is a member of the ZIP family of transporter and similar in sequence to a number of Arabidopsis and *T. caerulescens* ZIP transporters.

It has been speculated based on similarities in Zn/Cd uptake kinetics of TcZNT1 in yeast with *T. caerulescens* root Zn/Cd uptake (high affinity Zn and low affinity Cd uptake), that TcZNT1 may be involved in Zn uptake from the soil into the root and thus presumably shares a similar role as that of ScZRT1 (Pence et al, 2000). Analysis of the 1.1kb region upstream of the *TcZNT1* coding region revealed a putative E2F binding domain with the consensus E2F binding sequence, TTTCCCCT, located at -106 bp upstream from the *TcZNT1* start codon. This putative E2F binding sequence is highly similar to those previously reported for both plant and animal E2F proteins. To test if TcE2F1 and TcE2F2 proteins can interact with the putative E2F binding site in the *Tc*ZNT1 promoter, the Clontech Protein-DNA binding assay was conducted. In this assay, each TcE2F protein was tagged in frame to a small 6 kDa ProLabel reporter on the C terminus of the protein and expressed in HEK293 cells for 24 hours. A Prolabel reporter is a portion of an enzyme complex that can catalyze a chemiluminescent substrate and measure the amount of protein bound to a known DNA sequence. Crude protein was extracted from the HEK293 cells and used to test the interaction between the E2F element in the *ZNT1* promoter and the two TcE2F proteins. Testing for interaction of the *TcZNT1* promoter E2F binding element and the two E2F proteins from *T. caerulescens* revealed that TcE2F1 is not able to bind the E2F sequence from the *TcZNT1* promoter. However, as seen in Figure 3.5, the E2F motif from the

Figure 3.2: (A) Phylogenic tree of known E2F family members from *T. caerulescens*, *A. thaliana* and *O. sativa*. Alignment of *T. caerulescens* E2F1 (B) and E2F2 (C) with their closest homologs in *A. thaliana*, depicting known domains from other previously characterized plant E2F's, including the nuclear localization signal (NLS), DNA binding domain, leucine zipper, marked box domain, and retinoblastoma (Rb) binding domain. Alignment was performed based on the Clustal W method.

A



B

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AtE2F1 MSEEVP--QQ FPSSKRQLHP SLSSMKPPLV APGEYHRFDA AETRGGGAVA DQVVSDAIVI   60
TcE2F1 MSESQQDSEQ YPPSKRQYHP SVSSMKPPLV APGEYHRFDA AETRGGGAS- ----------   60

AtE2F1 KSTLKRKTDL VNQIVEVNEL NTGVLQTPVS GKGGKAKKTS RSAKSNKSGT LASGSNAGSP  120
TcE2F1 -DQLKRKTDM VNQIEEPNEL NTGVLQTPVS GKGGKPKKSS RSVKSNKTGT QASGSNAGSP  120
                                          NLS
AtE2F1 GNNFAQAGTC RYDSSLGLLT KKFINLIKQA EDGILDLNKA ADTLEVQKRR IYDITNVLEG  180
TcE2F1 GNNFAQA-TC RYDSSLGLLT KKFINLIKQA EDGILDLNKA ADTLEVQKRR IYDITNVLEG  180
                                DNA Binding Domain
AtE2F1 IGLIEKTLKN RIQWKGLDVS KPGETIESIA NLQDEVQNLA AEEARLDDQI RESQERLTSL  240
TcE2F1 IGLIEKTLKN RIQWKGLDVS KPGETVESIA NLQDEIQNLT AEEVKLDDQI RESQERLTSL  240
                                  Leucine Zipper
AtE2F1 SEDENNKRLL FVTENDIKNL PCFQNKTLIA VKAPHGTTLE VPDPDEAGGY -QRRYRIILR  300
TcE2F1 SEDENNKRFL FVTEDDIKNL PCFQNKTLIA VKAPHGTTLE VPDPDEAGGY PQRRYRIILR  300
                                Marked Box Domain
AtE2F1 STMGPIDVYL VSQFEESFED IPQADEPSNV PDEPSNVPDV PSNLPSTSGL PENHDVSMPM  360
TcE2F1 STMGPIDVYL VSQFEEKFEE IPNADEPS-- ---------- --NLPSTSGL PENQDVAMPM  360

AtE2F1 KEESTERNME TQEVDDTQRV YSDIESHDFV DGIMKIVPPD LDMGVDYWFR SEVGEVSITD  420
TcE2F1 EEDSSDKNME TQEVDDTQRV YSEIESHDFV DGIMKIVPPD LDMDVDYWLR SEVGEVSITD  420
                                          Rb Binding Domain
AtE2F1 MWPDESGPDW NQMITFDQDH AGPSDNKILE QPQTPSSPTP EESTATRSPT GS          472
TcE2F1 LWPNESGADW NQIVTFDQDH AGPS-NTTLE QPRTPLSPAP ENSTATKS-T GS          472
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C

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AtE2F2 MAATSNSGED PTLSYHHRSP FRFELLQSIS SSDPRYSSLT PSSTNRPFSV SQSLPNSQLS   60
TcE2F2 MAATSNSGED PVL-YNHRSP FRFELLQSLP PTDPRYSSQT PSSTNRPFSV SEPLPNAQ-S   60

AtE2F2 PLISPHWDDS YSQITQKVQK SRKNHRIQLG SIANMSGGES IDIAKVIVKQ ESSPQNVKRV  120
TcE2F2 PQIPPHWDES YSQITQKVQK SRKNHRIQLG SISNMPG-ET IDIAKVIVKQ ES-PQDTKRG  120

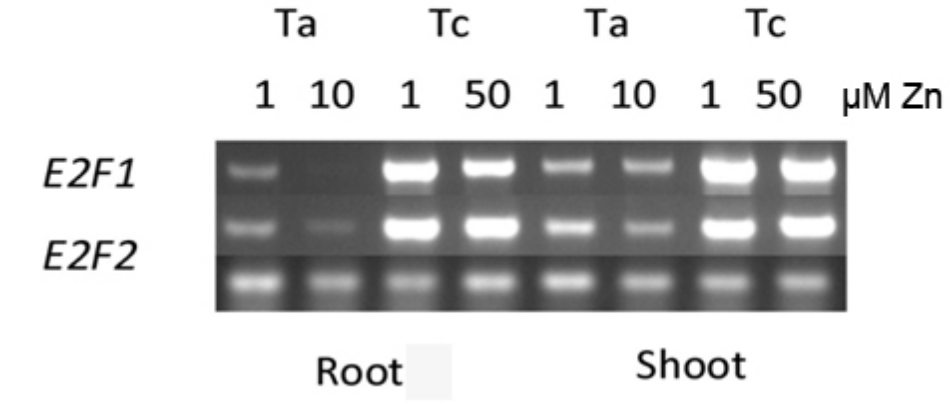
AtE2F2 YNKSKGGTKL LKAGKRMANG EVQNGGLNGA SINCRYDSSL GLLTKKFVKL IQEAEDGTLD  180
TcE2F2 -IKSK-VAKQ LKAGKRMANT EAHNGGPNGT N-NCRYDSSL GLLTKKFVNL IREAEDGSLD  180
          NLS                                  DNA Binding Domain
AtE2F2 LNYCAVVLEV QKRRIYDITN VLEGIGLIEK TTKNHIRWKG ADNLGQKDLG DQISRLKSEV  240
TcE2F2 LNYCADVLEV QKRRIYDITN VLEGIGLIEK TTKNHIRWKG ADNLGQNGLG DQISRLKLEV  240

AtE2F2 ESMQSEESRL DDLIRERQEA LRSLEEDYC RRYMFMTEED ITSLPRFQNQ TLLAIKAPTA  300
TcE2F2 ESMQSEESRL DELIRERQEA LRSLEEEYC KRYMFMTEED ITSIPRFQNQ TLLAIKAPTA  300
            Leucine Zipper                Marked Box Domain
AtE2F2 SYIEVPDPDE -MSFPQ-QYR MVIRSRMGPI DVYLLSKYKG DSAETSDKLG NESDQKAPVG  360
TcE2F2 SYIEVPDPDE VMSFPQRQYR MVIRSRMGPI DVYLLSKYKR DSGETS---- -ESDQNTQSG  360

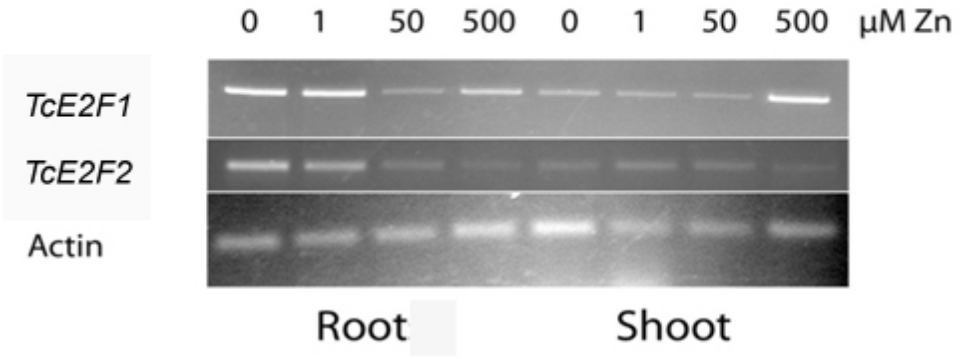
AtE2F2 VDTPSLKIVT SDTDLKADYW FESDAEVSLT DLWSNFNS 398
TcE2F2 VDTPSLKIVT SDTDLKTDYW FESDAEVSLT DLWNNNFS 398
                          Rb Binding Domain
```

Figure 3.3: A. Semi-quantitative RT-PCR analysis of *E2F1* and *E2F2* expression in both *T. arvense* and *T. caerulescens* plants grown on sufficient Zn (1μM) and high Zn (10 μM Zn for *T. arvense* and 50 μM Zn in *T. caerulescens*) in both roots and shoots. Cycle numbers were chosen to show the differences in expression and are not necessarily in the linear range of amplification. B. Semi quantitative RT-PCR analysis of *E2F1* and *E2F2* expression in *T. caerulescens* seedlings grown on 0, 1, 50 or 500 μM Zn for seven days, and then assayed for either *TcE2F1* or *TcE2F2* transcript abundance in root and shoot tissue.

A


Ta
Tc
Ta
Tc
1 10 1 50 1 10 1 50 µM Zn
E2F1
E2F2
Root
Shoot

B


0 1 50 500 0 1 50 500 µM Zn
TcE2F1
TcE2F2
Actin
Root
Shoot

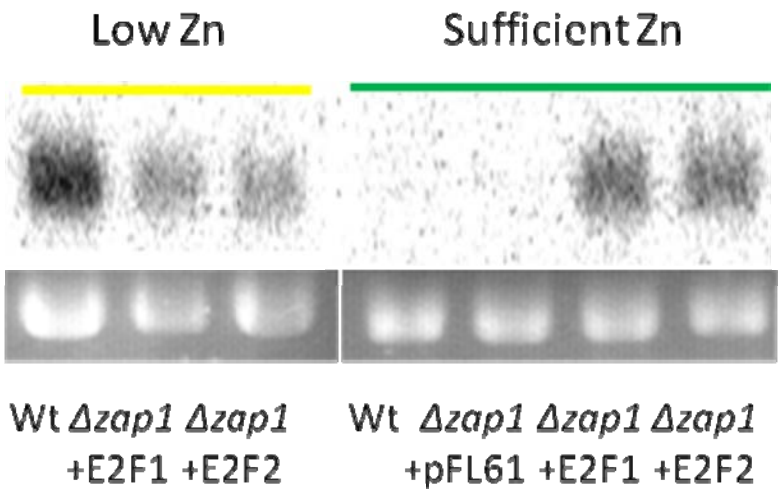


Figure 3.4: *TcE2F*-dependent yeast *ZRT1* expression in the *zap1*Δ yeast mutant background. Northern analysis for expression of the yeast high affinity Zn transporter, *ZRT1*, in the yeast Δ*zap1* mutant expressing *TcE2F1* and *TcE2F2* under the control of the phosphoglycerate kinase promoter in the pFL61 vector. Yeast was grown on SD media supplemented with 1 mM EDTA and 2 mM $ZnSO_4$ (High Zn), 1mM $ZnSO_4$ (Sufficient Zn) or 500 μM $ZnSO_4$ (Low Zn).

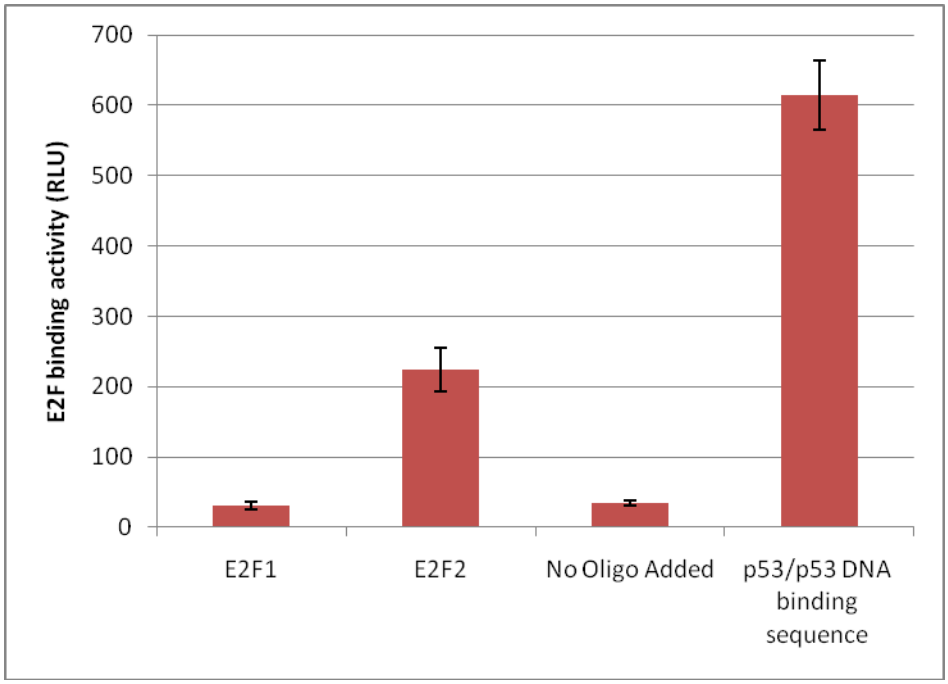


Figure 3.5: Quantification of TcE2F1 and TcE2F2's ability to bind to an E2F binding motif in the promoter of *TcZNT1*. The graph depicts the relative ability of either TcE2F1 or TcE2F2 -ProLabel fusions to bind to the biotinated oligo containing a 3X repeat of a consensus E2F element found in the promoter of *TcZNT1*. 'No oligo added" is the negative control to normalize all values for the absorbance for the enzyme mixture alone, and the p53/p53 binding sequence interaction serves as a known high affinity protein-DNA interaction for comparison. Values are based on replicates of three independent experiments and corrected for the amount of either E2F protein in the crude protein extract.

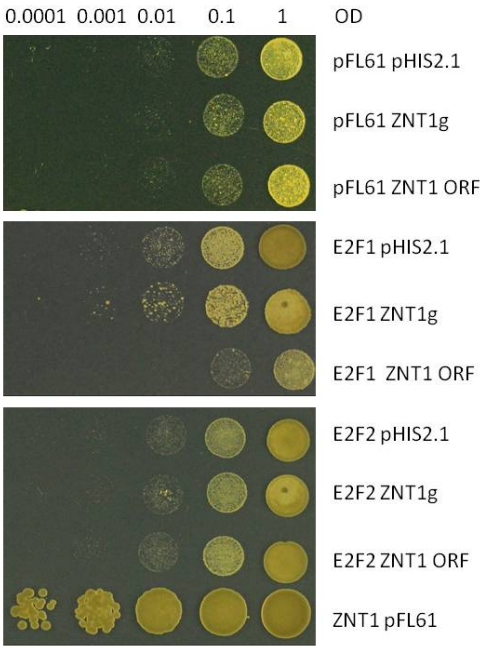


Figure 3.6: Directed yeast one hybrid to test for activation of *TcZNT1* expression by either TcE2F1 or TcE2F2 in the yeast double mutant, *ZHY3*, defective in both high and low affinity Zn uptake (*Δzrt1/zrt2*). The yeast one hybrid assay was run with yeast expressing either: the empty vector control (pFL61), *TcE2F1,* or *TcE2F2* in the yeast *ZHY3* mutant that was also co-expressing a genomic clone of *TcZNT1* driven by it's own promoter (1.1 kb region upstream of the start codon - labeled ZNT1g in the figure)), or the same genomic region with the introns removed (ZNT1 ORF). *TcZNT1* expressed in pFL61 (Pence et al., 2000) was used as a positive control. Yeast were grown for 72 hrs on Zn limited media (SC-URA + 500 μM ZnSO4 +1mM EDTA).

TcZNT1 promoter was able to be bound by the TcE2F2 protein.

To begin to investigate whether TcE2F1 and TcE2F2 can regulate or activate the expression of *TcZNT1*, a *TcZNT1* genomic fragment that spanned from 1.1 kb upstream of the start codon to the stop codon was cloned into the yeast expression vector pHIS2.1. Both *TcE2F* and the *TcZNT1* genomic region with its native promoter were co-expressed in the ZHY3 yeast mutant that lacks both yeast high and low affinity Zn transporters (*Δzrt1/zrt2*) and tested for growth on low Zn. Under low Zn conditions, neither TcE2F was able to activate ZNT1 expression and restore growth on Zn-limiting media (Figure 3.6). The possibility of improper splicing of the genomic region of *TcZNT1* in yeast could have occurred since *TcZNT1* contains two introns. A second clone was generated for the same *TcZNT1* genomic region without the introns and was also cloned into the pHIS2.1 vector and labeled as TcZNT1 ORF. Under low Zn conditions, neither TcE2F was able to activate expression of TcZNT1 when co-expressed with either *TcZNT1* genomic clone, and thus did not restore growth on Zn limiting media (Figure 3.6).

DISCUSSION

A number of published molecular investigations have been devoted to examining genes that may play a role in Zn uptake/storage in the Zn/Cd hyperaccumulators, *T. caerulescens* and *Arabidopsis halleri*. A number of the metal related genes in these studies exhibit higher levels of expression in the hyperaccumulator species compared with a related non-accumulator, and this increased expression has been shown to correlate with the higher Zn accumulation. It is well documented for “normal” non-accumulator plants that

a reciprocal relationship exists between internal ion concentrations and root ion influx (Glass and Siddiqi, 1984, Johansen et al., 1970; Lee, 1982), and for many mineral transporters it appears that this relationship is due to decreased expression of the transporter as the plant status for the mineral being transported increases from deficiency to sufficiency (for example, *AtZIP1, 2* and *3* and plant Zn status; Grotz et al., 1998). In the Zn/Cd hyperaccumulator species, many metal transporter and metal-related genes are expressed to high levels in the plant, even as the plant hyperaccumulates Zn, and a few recent publications have begun to look at this relationship (Becher et al., 2004; Hammond et al., 2006; Weber et al., 2004). In the case of *T. caerulescens* where it has been shown that the rate of Zn transport at a number of sites within the plant are greater than in non-accumulator species (Lasat et al., 1996; 1998), this increased Zn transport appears to be due in part, to the increased expression of metal transporters and other related genes. Also, because the relationship between metal transport and plant metal status may be somewhat different in hyperaccumulators compared with non-accumulator plant species, the possibility exists that the regulation of ion transport by plant nutrient status has been altered (see, Pence et al., 2000; Letham et al., 2005). However to date, no gene(s) have been identified that could be responsible for the underlying molecular mechanism for the altered Zn homeostasis and gene hyperexpression in *T. caerulescens.*

The control of metal uptake into roots is an important feature allowing plant survival in heavy metal contaminated soils. Previous published results indicate that in both Zn hyperaccumulator and non-accumulator species of *Thlaspi*, root Zn influx is regulated by plant Zn status. This regulation, which involves reduced root Zn influx as plant Zn status increases, is seen in both

Thlaspi species. However, in *T. caerulescens* the reduction in root Zn uptake is observed at much higher Zn levels (both outside and within the plant). Therefore, even at high levels of shoot and root Zn content, the greater Zn uptake and transport seen in *T. caerulescens* plants appears to be primarily achieved via Zn transporter gene expression that is not down regulated by very high levels of tissue Zn. Eventually, as we showed previously in Letham et al., (2005), when *T. caerulescens* is grown on high enough levels of Zn in the nutrient solution (50 to 500 μM), *TcZNT1* expression does decrease, but is still maintained at higher levels than the expression of the *ZNT1* homolog in *T. arvense*. Therefore, these findings suggest that the regulation of a Zn transporter gene expression plays an important role in the Zn hyperaccumulation phenotype of *T. caerulescens* and was the impetus for the research detailed here to begin to identify possible components of this regulatory scheme.

The identification of the transcription factors, TcE2F1 and TcE2F2, provide us with intriguing candidates for beginning to study the molecular basis for gene hyperexpression in *T. caerulescens*. Because the genes encoding these E2Fs were also hyperexpressed in *T. caerulescens*, it is possible that elevated levels of the E2F proteins in *T. caerulescens* could be involved in the higher expression of the heavy metal-related genes described in this study. Complementation of the yeast *zap1*Δ mutant with both TcE2F1 and TcE2F2 restored yeast growth on low Zn media via activation of expression of the yeast high affinity transporter, ZRT1. Also, we were able to show that of the TcE2F2 protein binds to a consensus E2F binding motif with high affinity in the promoter of *TcZNT1*. These findings provide intriguing circumstantial results that TcE2F2 might play a role in the regulation of gene

expression associated with elevated Zn uptake and transport in *T. caerulescens*. However, the next critical step is to obtain direct *in planta* evidence that TcE2F regulates Zn transporter expression. Certainly the directed yeast 1-hybrid results in Figure 3.6, where when both TcE2F and *TcZNT1* were expressed in yeast but failed to activate *TcZNT1* expression, suggest that in yeast, other higher plant proteins may be required for *bona fide* activation of Zn transporter gene expression. Furthermore, the inability of TcE2F1 to bind the E2F element in the *TcZNT1* promoter suggests a different role for TcE2F1 either in Zn homeostasis or regulation of other metals in *T. caerulescens*.

While a small number of studies have examined the function of the E2F proteins in plants, most have focused on characteristics of E2Fs previously identified from work in animal systems. The E2F family of transcription factors has been shown to be involved in cell division and cell growth mainly via regulation of the transition from G_1 to S phase of the cell cycle (Albani et al, 2000; Mariconti et al., 2002). This role for E2Fs is fairly well characterized in animal systems and a number of studies in Arabidopsis have indicated a similar role for AtE2Fs. It is not unprecedented that transcription factors can serve dual roles. In fact, this is not the first time it has been suggested that the regulation of mineral nutrition homeostasis involves transcription factors that are also involved in regulation of the cell cycle. The yeast cyclin dependant protein kinase, PHO85, was once only thought to only be involved in the control of phosphate uptake under times of limiting inorganic phosphate. However more recent studies on PHO85 have revealed other roles for this protein in glycogen synthesis and cell cycle progression (Pringle and Hartwell, 1981: Timblin, 1996). As yeast has been a useful model system for the study

of molecular mineral nutrition for a number of mineral nutrients in plants, including Zn, Fe, and P, it is reasonable to speculate that a set of transcription factors involved in the plant cell cycle may also play a role in plant mineral homeostasis. The findings presented here are both intriguing and puzzling. We were able to show that two related plant transcription factors, TcE2F1 and TcE2F2, were able to activate expression of the gene encoding the yeast high affinity Zn uptake transporter, *ZRT1*. However, it was interesting to note that the TcE2Fs did so in a manner independent of yeast Zn status, in that *ZRT1* expression was activated to similar levels in low Zn and sufficient Zn-grown yeast (Figure 3.4). This may be due to the constitute expression of both TcE2F proteins, respond to changes in Zn status, with expression increasing as status changes from high to low Zn. These findings indicate that the TcE2Fs cannot completely replace the function of ZAP1 in yeast, and/or other yeast proteins are involved in regulation of *ZRT1* gene expression. Also, it was interesting to see that the E2Fs, when co-expressed in yeast with the *T. caerulescens ZRT1* homolog, *TcZNT1*, failed to activate expression of *TcZNT1*. One obvious explanation is that in plants, other proteins may function in conjunction with the TcE2Fs to regulate *TcZNT1* expression. Also, it may be that *TcZNT1* may not be the best candidate metal transporter for interaction with and regulation by TcE2F1 or 2. Hence we will now take a broader, *in planta* approach. This will first involve studying regulatory interactions between TcE2Fs and *TcZNT1* using plant-based transient expression systems. Furthermore, we have found that a number of Arabidopsis ZIP family members harbor the same putative E2F binding motif that exists in the *TcZNT1* promoter that we showed here binds TcE2F2 with relatively high affinity (Figure 3.5). It could be that different members of the E2F family interact with different metal

transporters. Circumstantial evidence in support of this comes from our preliminary findings that another E2F, AtE2F3, appears to play a role in regulation of Fe uptake when expressed in yeast (Milner and Kochian, unpublished results).

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MATERIALS AND METHODS

Plant Material

Thlaspi caerulescens ecotype Prayon and *Thlaspi arvense* seedlings were grown hydroponically in nutrient solution containing 1 μM Zn^{2+}. See Lasat et al. (1996) for details concerning the composition of the nutrient solution. As *T. arvense* seedlings grow somewhat more rapidly than *T. caerulescens*, seedlings of the two *Thlaspi* species were grown for different lengths of time to yield plants at similar developmental stages, in a greenhouse, as described previously (Lasat et al., 1996; Lasat et al, 1998). Following this initial growth period, the nutrient solution was supplemented with different Zn^{2+} concentrations ranging from 0 to 500 μM for *T. caerulescens* and 0 to 20 μM for *T. arvense.* Solutions were replaced every five days.

RNA extraction

Plants were pooled from 2.2L pots with 4 plants in each pot grown on Zn sufficient or high Zn nutrient solutions for each species; this was repeated four separate times. For first stand synthesis for RT-PCR, RNA was extracted using Trizol, according to the manufacturer's instructions **(**Invitrogen, Carlsbad, CA, USA**).** RNA was then run through a Qiagen Mini prep RNA column (Qiagen Inc., Valencia, CA, USA) to further purify each RNA sample.

Semi Quantitative RT-PCR

Equal amounts (500 ng) of total RNA was used for each reaction in Invitrogen's One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). First strand synthesis was carried out at 45^{o} C for 1 hr and then 94^{o} C for 2 min to stop and denature the reverse transcriptase. This was immediately followed by a denaturing step at 94^{o} C for 15 sec, an annealing step at 58^{o} C for 30 sec, and finally an extension step estimating that the time for one kb of extension was one minute. Amplification products were then cloned into pGEM Easy T vector **(**Promega, Madison ,WI, USA **)** and sequence was verified to ensure correct product.

***Thlaspi* cDNA expression in yeast**

Primers for conserved regions of other known E2F1 and E2F2 nucleotide sequences for various plant species were used to clone a fragment of TcE2F1 and TcE2F2 (Invitrogen, Carlsbad, CA, USA). Rapid Amplification of cDNA ends was then performed to obtain a full length cDNA. Full length clones were ligated into the bifuncional yeast/Escherichia coli expression plasmid vector, pFL61, using the BstXI/EcoRI sites (Minet et al, 1992). The TcE2F1 and TcE2F2 cDNAs were then transformed into the yeast ZHY6 mutant background, the transformed yeast were cultured under both low and high levels of Zn and allowed to grow for 48–72 hrs to assess restoration of growth. Low Zn treatment consisted of supplemented minimal media amended with 0.1% casamino acids and all necessary auxotrophic supplements except for uracil, 10 µM Fe-EDTA, and a zinc chelate made from 500 µM $ZnSO_4$ and

1mM EDTA. High Zn media contained the same as the low Zn media except for the addition of 2mM $ZnSO_4$ and 1mM EDTA

E2F1 and E2F2 expression in yeast

Northern analysis was performed to assay for the expression of the yeast high affinity Zn transporter, *ZRT1*, in the yeast Δ*zap1* mutant expressing *TcE2F1* or *TcE2F2* under the control of the phosphoglycerate kinase promoter in the pFL61 vector. Yeast were grown on SD media with zinc chelates made with 1 mM EDTA and 2 mM $ZnSO_4$ (High Zn), 1mM $ZnSO_4$ (Sufficient Zn) or 500 □M $ZnSO_4$ (Low Zn). Northern analysis was performed on total RNA isolated from high Zn, sufficient Zn and low Zn grown yeast. Ten µg of total RNA was transferred to a nylon membrane (Hybond N^+; Amersham Pharmacia) and probed with labeled ScZRT1. The membranes were then washed under high stringency twice in a solution containing 0.2X SSC, 0.1 % SDS at 65^o C.

Yeast One Hybrid

TcE2F1 and TcE2F2 originally isolated from the yeast complementation screen were isolated from the ZHY6 yeast background to later be transformed into the ZHY3 background. The ZNT1 genomic region was amplified using the primers cgatcgGACTGAAGATGGCA and gagctcCTAAGCCCAAATGGCGAGT into pGEM easy T vector and digested with SacI and PvuI to release the insert and cloned into the pHIS vector with the corresponding sites. A fusion of the ZNT1 promoter and TcZNT1 ORF were fused by amplyifing the promoter and partially into the open reading frame of *TcZNT1* and the 3' end of the ORF using the primers

cgatcgGACTGAAGATGGCA,
TTCGTGGGAGATGAAGCCATTGTCTGCAAAATGGACT.
For the promoter region the primers
AGTCCATTTTGCAGACAATGGCTTCATCTCCCACGAA and
gagctcCTAAGCCCAAATGGCGAGT were used. The products of these two PCRs were mixed and then amplified using the primers used to isolate the genomic clone and cloned into pGEM Easy T. ZHY3 yeast cells were then grown overnight in YPG and transformed with either pFL61, TcE2F1 or TcE2F2 and plated on SC-URA plates to select for transformants. Transformed colonies were picked into a liquid culture of SC-URA and grown overnight and transformed with either pHIS2.1, TcZNT1 genomic region or TcZNT1 promoter and ORF and selected for on SC-URA-TRP plates.

Quantification of E2F binding to ZNT1 E2F motif

TcE2F1 and TcE2F2 were cloned into the pProLabel C vector (Clonetech, Mountain View, Ca) using the EcoRI/KpnI cloning site to create an in-frame fusion of Pro Label and the E2F proteins. The recombinant plasmids containing either TcE2F1 or TcE2F2 were transfected into the human kidney cell line HEK273 and grown for 48 hours. Crude protein was isolated from the cell culture and used for subsequent assays. Fifty µL of crude extract was incubated with a biotinated 3X repeat of the ZNT1 E2F sequence or a mutated form of the ZNT1 E2F sequence changing the fifth base pair from a C to an A. Crude Protein was then added to a streptavin plate and washed four times to remove any non-specific protein DNA interactions. Wells were then assayed for luciferase activity using a Biotech Synergy HT plate reader (Winooski,

Vermont). Signal was normalized based on crude extract activity to compensate for amount of recombinant protein in each crude extract.

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CHAPTER IV

Ecotypic differences in metal hyperaccumulation in *Thlaspi caerulescens*: Searching for genes that contribute to metal hyperaccumulation and tolerance in plants

ABSTRACT

There is growing evidence for the Zn/Cd hyperaccumulator *Thlaspi caerulescens*, that Zn and Cd hyperaccumulation involves different transport and tolerance mechanisms. It has been well documented that certain ecotypes of *T. caerulescens*, especially those like the Ganges ecotype from the south of France, are much better Cd hyperaccumulators than ecotypes such as Prayon. However, there does not seem to be much ecotypic variation in Zn hyperaccumulation in *Thlaspi*. In this study we took a comparative transcriptomics approach using the Affymetrix ATH1 gene chip to look at shoot gene expression in the ecotypes Ganges and Prayon in response to Cd stress. The goal of this study was to identify genes that were more highly expressed in shoots of the superior Cd accumulator, Ganges, which might be candidates in Cd hyperaccumulation and tolerance. From this microarray screen, we identified the vacuolar metal transporter, HMA3, as one of the more highly expressed genes in Ganges shoots; where a roughly twofold higher level of expression was seen in Ganges as compared to Prayon following exposure to cadmium. These microarray results were verified using quantitative real-time PCR which showed that *TcHMA3* was more highly expressed in both roots and shoots of the Ganges ecotype in response to Cd. *HMA3* was cloned from both *Thlaspi* ecotypes and Arabidopsis and was found to encode a membrane transporter localized to the tonoplast in leaf cells.

Hypothesizing that these transporters may explain aspects of the differential metal hyperaccumulation displayed by these two ecotypes, we expressed the respective *TcHMA3* ortholog from each ecotype as well as *AtHMA3* in yeast to compare their transport characteristics. From these studies we found that both TcHMA3 orthologs as well as AtHMA3 were effective Cd uptake transporters, presumably functioning to accumulate Cd in the cell vacuole. Additionally, studies in yeast also indicated that AtHMA3 had a much better capacity to transport Zn, Cu and Pb. Both *TcHMA3* orothologs were expressed in transgenic Arabidopsis seedlings which resulted in significant increases in root Cd tolerance and accumulation. Therefore, we hypothesize that TcHMA3 is a player in Cd hyperaccumulation in the shoots of both ecotypes of *T. caerulescens* and is the first vacuolar transporter in *T. caerulescens* shown to transport Cd.

INTRODUCTION

The zinc (Zn)/cadmium (Cd) hyperaccumulating model species *Thlaspi caerulescens* J.Presl & C.Presl (= *Noccaea caerulescens*) has served as an excellent model for the study of heavy metal hyperaccumulation in plants. While Zn influx and translocation in *T. caerulescens* have been extensively studied, the ability to transport and tolerate Cd is, compared to most “normal“ plants, even more striking (Pence et al 2000; Küpper et al 2004.; Lombi et al., 2001, Lasat et al., 2000). Leaf Cd concentrations of up to 10,000 mg kg DW^{-1} have been observed without any indication of toxicity, whereas the typical shoot concentration in plants is between 0.1–10 mg Cd kg DW^{-1} (Kabata-Pendias and Pendias, 2000). This phenotype involves increased uptake and translocation to the aerial portions of the plant coupled with highly efficient

mechanisms that provide Cd tolerance at the leaf level, typically through sequestration in the leaf cell vacuole (Ma et al., 2005). Of considerable current interest is the identification of the genetic basis of these traits.

A number of studies have examined the path by which Cd enters and is transported throughout hyperaccumulating plants (Cosio et al., 2004; Ebbs et al., 2009; Küpper et al., 1999, 2004, 2007; Ma et al., 2005; Ueno et al., 2005). There is considerable intraspecific variation between populations of *T. caerulescens* in the capacity to transport and tolerate Cd. Comparison of the two most widely studied ecotypes of *T. caerulescens* has shown that the ecotype referred to as Prayon was considerably less tolerant to Cd than a Southern France population typically referred to as Ganges (Cosio et al, 2005; Lombi, et al, 2001; 2002 Zha, et al., 2004). While both are still considered Cd hyperaccumulators, the Ganges ecotype showed a fivefold higher V_{max} for unidirectional influx into roots as compared to the Prayon ecotype, but no difference in the K_M value for influx between the ecotypes was seen (Lombi et al., 2001). When plants were grown under Fe limiting conditions a threefold increase in root Cd influx could be seen in Ganges, but no change was observed in Prayon (Lombi et al., 2002). In isolated protoplast from leaves, the concentration dependent kinetics of unidirectional Cd flux in Prayon protoplasts had a two-fold higher V_{max} than in Ganges leaf protoplasts with no difference in K_M. In contrast, comparison of Zn influx into roots showed negligible differences in kinetic parameters between the two ecotypes, which suggested that Cd is not moving into the plant on the same transporter as Zn (Lombi et al., 2001; Zhao et al., 2002; Zha et al., 2004). However the kinetics of root Cd uptake suggests that there is increased abundance of a transporter(s) involved in uptake is higher in the Ganges ecotype in the roots,

but not the shoots. The underlying gene involved in Cd uptake seen in the leaves suggests a transporter is involved and not a channel. While what the underlying gene transporting the Cd has yet to be elucidated.

In leaves of *T. caerulescens*, vacuolar sequestration is the main mechanism for metal tolerance (Cosio et al., 2005; Ebbs et al., 2009; Küpper et al., 1999, 2004, 2007; Ma et al, 2005; Ueno et al., 2005). The transporter encoded by *TcMTP1* (*ZTP1*) has been proposed to be a major contributor to Zn accumulation in the vacuole of cells in the leaf and therefore also involved in the hyperaccumulation demonstrated by this species (Assunção et al., 2001). *TcMTP1* is expressed mainly in the shoots of *T. caerulescens* and shows a low level of expression in root tissue. It also appears that there may be more than one copy of *TcMTP1* in the *Thlaspi* genome (Assunção et al., 2001). To date no tonoplast targeted protein(s) have been shown to transport Cd in *Thlaspi.*

There are several gene families that encode transporters that contribute to heavy metal tolerance in plants. Examples include the members of the CAtion eXchange (CAX) family, Natural resistance and macrophage protein family (Nramp), and Heavy Metal Associated (HMA) families (Pittman et al., 2004; Curie et al., 2000, Gravot et al., 2003; Morel et al., 2009). Recently in Arabidopsis *AtHMA3*, a P_{1B}-type ATPase, was shown to be vacuolar localized and to mediate the transport of Cd (Gravot et al., 2003; Morel et al, 2009). However yeast cells over expressing *AtHMA3* showed no significant difference in Cd accumulation but did exhibit higher Cd tolerance when Cd was added to the growth media (Gravot et al., 2003). In plants, *AtHMA3* overexpressing lines conferred tolerance to high levels of Zn, Cd and Pb in the growth media

and plants showed increased biomass when grown in the presence of Cd (Morel et al, 2009).

The study reported here investigated the underlying genetic basis for the differences in Cd transport and tolerance in the Prayon and Ganges ecotypes of *T. caerulescens*. The Cd-responsive transcriptomes of the two ecotypes were compared using microarray analysis. Based on observed differences in the expression of *HMA3* between the two ecotypes, with the Gange *HMA3* ortholog being more highly expressed, the *HMA3* orthologs were cloned from these two ecotypes and also from Arabidopsis. The expression and transport properties of the encoded proteins were characterized. The results demonstrate that *TcHMA3* is a tonoplast transporter that mediates the transport of Cd and is involved in the sequestration of Cd in both root and shoots of *T. caerulescens*.

RESULTS

Microarray experiment

Similar to the approach used by Schat and Kalff (2002) in their study of the role of phytochelatins in metal tolerance in metallophytes, the strategy employed here to identify genes involved in Cd tolerance sought to compare gene expression in Prayon and Ganges plants exposed to solution Cd concentrations that produced a comparable physiological effect in those plants. To determine this ecotype-specific treatment concentration, it was necessary first to identify the concentration that caused Cd phytotoxicity in each ecotype over a reasonably short time period (seven days) in order to capture changes in gene expression in response to the early stages of Cd stress. For the Prayon ecotype of *T. caerulescens*, there were no adverse

effects noted following exposure to 0.1 or 0.5 mM $CdSO_4$ in full nutrient solution for seven days, but plants became obviously chlorotic at 1 mM or higher, with the severity of the chlorosis increasing with Cd concentration. Ganges plants showed a similar degree of chlorosis at 5 and 10 mM $CdSO_4$ yet showed no symptoms at concentrations of 1 mM or lower. Leaf tissues from plants exposed to the highest concentration tested that did not impose Cd phytotoxicity on a given ecotype (0.5 mM for Prayon, 1.0 mM for Ganges) were subjected to elemental analysis. Leaf Cd concentrations exceeded 16,100 mg kg DW^{-1} in the Ganges ecotype compared to ~790 mg kg DW^{-1} in Prayon, concentrations indicative of Cd hyperaccumulation by each ecotype (Figure 4.1). Consequently, the corresponding solution Cd concentrations were used to impose the Cd treatment on plants for the microarray experiment.

Total RNA from the Prayon or Ganges ecotype was hybridized to the ATH1 Affymetrix chip to compare the transcriptomes in response to Cd treatment. Since there is not a genome wide microarray available for *T. caerulescens*, the Affymetrix ATH1 genome chip was used due to the high degree of homology between the *Thlaspi* and Arabidopsis genomes. *Thlaspi caerulescens* and *Arabidopsis thaliana* share between 85-90% homology at the genomic sequence level in the coding regions (Peer et al., 2003). The probes for the microarray were derived from a similar transcriptome study (Hammond et al. 2005) that hybridized *T. caerulescens* genomic DNA to the same ATH1 microarrays to call the proper match oligo in each probe set, and the rest of the mismatch oligos in each probe set for background subtraction. While this most likely does not cover the full *T. caerulescens* genome, it is estimated to provide over 22,000 unique probes.

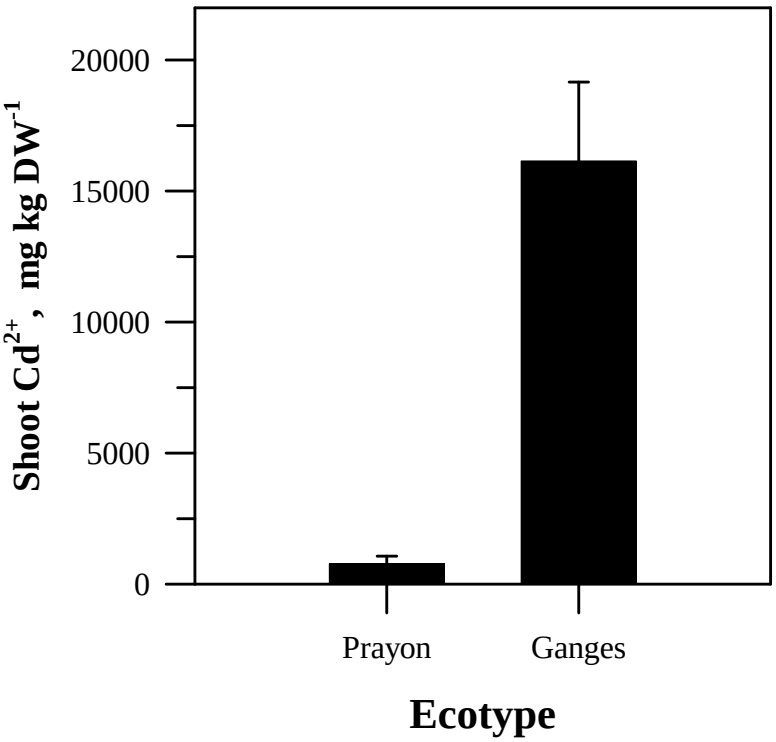

Figure 4.1: Concentration of Cd in the shoots of the Prayon and Ganges ecotypes of *T. caerulescens* following a 7 d exposure to 0.5 or 1 mM $CdSO_4$, respectively. Data represent the mean and standard error (*n*=4).

Based on the comparison of the two shoot transcriptomes, a total of 135 genes showed higher expression in Ganges as compared to Prayon under Cd stress, with 21 of the 135 genes encoding transporters (Table 4.1). In Prayon, a total of 139 genes were expressed higher than in Ganges, including 18 transporters (Table 4.2). Of the 21 transporter genes expressed higher in Ganges, three genes encoding tonoplast transport proteins were more highly expressed, and of these three genes, *TcHMA3*, was selected for further study based on current literature suggesting a role for *AtHMA3* in Cd transport at the tonoplast (Gravot et al., 2003; Morel et al, 2009). From the microarray study, *TcHMA3* showed an approximately two-fold increase in expression in shoots of the Ganges ecotype compared to the Prayon ecotype (FDR 0.08691).

Cloning *TcHMA3*

TcHMA3 gene was cloned from Prayon and Ganges plants using total RNA isolated from plants exposed to 100 µM Cd for seven days. The gene was first cloned from the Prayon ecotype, and once a full length clone was isolated, the same primers were used to isolate the Ganges ortholog of *HMA3*. A comparison of the cloned *TcHMA3* genes revealed that there were two different loci encoding the same protein for *TcHMA3* in *T. caerulescens*. The open reading frame of each paralog is identical between the two respective copies in their respective genomes. The paralogs in Prayon encode identical 759 amino acid proteins and in Ganges, the paralogs encode identical 757 amino acid proteins. Comparison of the coding sequences for the two different orthologs of *TcHMA3* to *AtHMA3* show that the *Thlaspi* versions share greater than 99 percent identity over the 2.3 kb open reading frame,

Table 4.1: Transporter genes that show higher expression in the shoots of Ganges versus Prayon treated with 1mM or 0.5 mM Cd, respectively. Significance was determined using a FDR cut off of 0.1

Probe ID	Ratio	FDR	Annotation	AGI Code
261895_at	0.2715	0.00063196	metal ion transporter	At1g80830
244912_at	0.2973	0.00319808	cytochrome c biogenesis	AtMg00830
245846_at	0.3048	0.00257513	P-type transporting ATPase	At1g26130
258331_at	0.3083	0.0979124	putative coatomer complex subunit	At3g15980
252328_at	0.3354	0.0119544	Expressed protein	At3g48570
254305_at	0.3571	0.00955414	potassium channel protein AKT3	At4g22200
266963_at	0.3614	0.00830112	unknown protein	At2g39450
257338_s_at	0.3733	0.074332	NADH dehydrogenase subunit 5 (nad5)	AtMg00513
250712_at	0.3953	0.0185386	human RAN binding protein 16-like	At5g06120
246566_at	0.4145	0.0996611	oligopeptide transporter -like protein oligopeptide transporter (LeOPT1)	At5g14940
264520_at	0.4178	0.0301935	putative amino acid permease	At1g10010
260481_at	0.4301	0.00682567	ferredoxin precusor isolog	At1g10960
249535_at	0.4436	0.0314292	transporter -like protein N system amino acids transporter NAT-1	At5g38820
260002_at	0.4442	0.0893862	putative ABC transporter	At1g67940
248062_at	0.4524	0.02166	unknown protein	At5g55450
246862_at	0.4779	0.0329509	E2, ubiquitin-conjugating enzyme	At5g25760
253658_at	0.5092	0.0869102	cadmium-transporting ATPase-like protein E1-E2 cadmium efflux	At4g30120
253172_at	0.5241	0.0444772	putative protein	At4g35060
248756_at	0.5559	0.0988234	sodium-dicarboxylate cotransporter-like	At5g47560
256224_at	0.5816	0.0767234	GTP-binding protein (SAR1B)	At1g56330
256305_at	0.601	0.0951487	glutathione S-conjugate transporting ATPase (AtMRP1)	At1g30400

Table 4.2: Transporter genes that show higher expression in the shoots of Prayon versus Ganges treated with 0.5 mM or 1 mM Cd, respectively. Significance was determined using a FDR cut off of 0.1

Probe ID	Ratio	FDR	Annotation	AGI Code
261532_at	1.6739	0.0893734	amino acid permease, putative similar to lysine and histidine specific transporter	At1g71680
257842_at	1.7142	0.0754345	P-glycoprotein	At3g28390
261806_at	1.715	0.0677748	ferrodoxin NADP oxidoreductase	At1g30510
252215_at	1.9709	0.034621	kinesin -like protein KINESIN-LIKE PROTEIN KIF4	At3g50240
246219_at	1.9951	0.0664662	aminopeptidase-like protein	At4g36760
261563_at	2.1338	0.0265191	polyphosphoinositide binding protein	At1g01630
248849_at	2.295	0.0996611	multidrug resistance p-glycoprotein	At5g46540
249158_at	2.3391	0.0136863	electron transfer flavoprotein beta-subunit-like	At5g43430
251106_at	2.3591	0.0860429	putative protein peroxisomal Ca-dependent solute carrier	At5g01500
265476_at	2.5017	0.0301935	putative thioredoxin M	At2g15570
258345_at	2.6576	0.0532851	unknown protein	At3g22845
248221_at	2.7187	0.0112518	vacuolar sorting protein-like	At5g53530
255442_at	2.7947	0.0381333	NADH-ubiquinone oxidoreductase 24 kDa subunit	At4g02580
248512_at	3.962	0.00173196	protein translocation complex Sec61 gamma chain	At5g50460
256308_s_at	4.323	0.0112009	ABC transporter	At1g30410
248392_at	7.9071	0.00346561	integral membrane protein-like	At5g52050
260281_at	9.5491	0.00134766	unknown protein	At1g80500
246302_at	11.8626	0.00015465	Ca2+/H+-exchanging protein-like Arabidopsis thaliana high affinity calcium antiporter CAX1	At3g51860

while the two *Thlaspi* ortholog compared to AtHMA3 reveals a sequence similarity of 89.2 and 89.1 percent. At the amino acid level, the orthologs show 96.7 percent identity and 86.8 (Prayon HMA3) and 87.6 percent (Ganges HMA3) identity when compared to AtHMA3 (Figure 4.2).

Transcript Abundance

To better understand the expression pattern of *HMA3* in roots and shoots of *T. caerulescens* in the absence or presence of low and high Cd levels, relative transcript abundance was determined using quantitative real-time PCR. Primers were designed to detect expression of both paralogs in Prayon on Ganges. In Ganges roots, *HMA3* expression was fourfold higher than in Prayon roots (Figure 4.3). In the roots of Prayon, *HMA3* transcript abundance significantly increased as the Cd concentration increased, yet the transcript levels were still less than those in roots of both low Cd-treated and untreated Ganges roots. There was no significant difference in transcript abundance in Ganges roots in untreated and low Cd treated roots, while a significant increase in *HMA3* expression was seen when Ganges roots were treated with high levels of Cd. In the shoots of either untreated or low Cd-treated plants, there was a slightly lower level of *HMA3* expression in Prayon compared to in Ganges, but this difference was not statistically significantly. At high levels of Cd, there was no significant change in *HMA3* transcript levels in the shoots of Prayon, while in Ganges there was a significant increase in shoot *HMA3* expression in response to high Cd.

Yeast Data

The specificity of the HMA3 transporter for divalent cations was tested in wild type yeast constitutively expressing each HMA3 ortholog. When either HMA3 ortholog was expressed in WT yeast, there was a significant increase in Cd accumulation compared with yeast expressing the empty vector ($p \leq 0.0001$), and the HMA3 orthologs from each ecotype were equally effective in transporting Cd (Figure 4.4). Expression of *AtHMA3* in yeast resulted in a highly significant ($p \leq 0.0005$) increase in yeast Cu, Pb and Zn accumulation, while the orthologs from the two *Thlaspi* ecotypes were not effective in transporting these three metals.

Subcellular Localization of TcHMA3

As AtHMA3 has been previously localized to the tonoplast in Arabidopsis (Morel et al., 2008), C-terminal GFP fusions proteins were developed for each TcHMA3 protein and transiently expressed in *Arabidopsis* to determine if the *Thlaspi* proteins were also associated with the tonoplast membrane. The characteristic wrapping around the chloroplast seen in figure 4.5 E and I are an indication of tonoplast localization. This suggests that the in-frame fusions of each of the two HMA3 proteins from each *Thlaspi* ecoptype were found to be tonoplast localized (Figure 4.5).

Over Expression *in Planta*

The two orthologs of *TcHMA3* were over-expressed in the Col-0 ecotype of Arabidopsis to determine whether constitutive expression of *TcHMA3* could increase Cd tolerance and accumulation. When transgenic Arabidopsis plants

Figure 4.2: Alignment of the amino acid sequence of AtHMA3 with the two orthologs from Ganges and Prayon. Alignment was performed using Clusta W. Highlighted sequence represents conserved amino acids between the AtHMA3 and either ortholog of TcHMA3.

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TcHMA3G   MKAIGEEATK KNVKTSYF-D VVGICCSSEV SIVGDVLRPL DGVIDFSVIV VSRTVIVVHD   60
TcHMA3P   MKAIGEEATI EECEDEVTPD VVGICCSSEV STVGDVLRPL DGVIDFSVIV VSRTVIVVHD   60
AtHMA3    M-AEGEESKK MNLQTSYF-D VVGICCSSEV SIVGNVLRQV DGVKEFSVIV PSRTVIVVHD   60

TcHMA3G   TLLISPLQIV KALNQARLEA SVRPYGETSL KSQRPSPFAV ASGVLLALSF LKYLYSPLAL  120
TcHMA3P   TLLISPLQIV KALNQARLEA SVRPYGETSL KSQRPSPFAV ASGVLLALSF LKYLYSPLAL  120
AtHMA3    TFLISPLQIV KALNQARLEA SVRPYGETSL KSQWPSPFAI VSGVLLVLSF FKYFYSPLEW  120

TcHMA3G   LAIVAVVAGI YPILAKAFAS VIRFRLDINA LTLIAVIATL CMQDYAESAT IVFLFSVADW  180
TcHMA3P   LAIVAVVAGI YPILAKAFAS VIRFRLDINA LTLIAVIATL CMQDYAESAT IVFLFSVADW  180
AtHMA3    LAIVAVVAGV FPILAKAVAS VTRFRLDINA LTLIAVIATL CMQDFTEAAT IVFLFSVADW  180

TcHMA3G   LESSAAHKAS TVMSSLLSLA PRKAVIAETG QEVDVDEVGI NTVVSVKAGE SIPIDGVVVD  240
TcHMA3P   LESSAAHKAS TVMSSLLSLA PRKAVIAETG QEVDVDEVGI NTVVSVKAGE SIPIDGVVVD  240
AtHMA3    LESSAAHKAS IVMSSLMSLA PRKAVIADTG LEVDVDEVGI NTVVSVKAGE SIPIDGVVVD  240

TcHMA3G   GSCDVDEKTL TGESFPVSKQ KDSTVLAATI NLNGYIKVKT TALAKDCVVA KMTKLVEEAQ  300
TcHMA3P   GSCDVDEKTL TGESFPVSKQ KDSTVLAATI NLNGYIKVKT TALAKDCVVA KMTKLVEEAQ  300
AtHMA3    GSCDVDEKTL TGESFPVSKQ RESTVMAATI NLNGYIKVKT TALARDCVVA KMTKLVEEAQ  300

TcHMA3G   KSQTQTQRFI DRCSRYYTPA VVLVAASFAL IPFLLKVQNL RHWFYLALVV LVSGCPCGLI  360
TcHMA3P   KSQTQTQRFI DRCSRYYTPA VVLVAASFAL IPFLLKVQNL RHWFYLALVV LVSGCPCGLI  360
AtHMA3    KSQTKTQRFI DKCSRYYTPA VVVSAACFAV IPVLLKVQDL SHWFHLALVV LVSGCPCGLI  360

TcHMA3G   LSTPVATFCA LTKAATSGFL IKTGDCLETL AKIKITAFDK TGTITKAEFT VSDFRSLSHN  420
TcHMA3P   LSTPVATFCA LTKAATSGFL IKTGDCLETL AKIKITAFDK TGTITKAEFT VSDFRSLSHN  420
AtHMA3    LSTPVATFCA LTKAATSGFL IKTGDCLETL AKIKIVAFDK TGTITKAEFM VSDFRSLSPS  420

TcHMA3G   INLHKLLYWV SSIESKSSHP MAAALIDYAR STSVEPKPDL VENFQNFPGE GVYGRIDGQD  480
TcHMA3P   INLHKLLYWV SSIESKSSHP MAAALIDYAR STSVEPKPDL VENFQNFPGE GVYGRIDGQD  480
AtHMA3    INLHKLLNWV SSIECKSSHP MAAALIDYAI SVSVEPKPDI VENFQNFPGE GVYGRIDGQD  480
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Figure 4.2 (Continued)

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TcHMA3G   IYIGNKRIAQ RAGCST--VP DIGANMKRGK TIGYIYIGQE LIGSFNLLDG CRHGVAQALE  540
TcHMA3P   IYIGNKRIAQ RAGCST--VP DIGANMKRGK TIGYIYIGQE LIGSFNLLDG CRHGVAQALE  540
AtHMA3    IYIGNKRIAQ RAGCLTDNVP DIEATMKRGK TIGYIYMGAK LTGSFNLLDG CRYGVAQALK  540

TcHMA3G   ELKSLGIKTA MLTGDNRDAA MSTQEQLGNA LDIIHSELLP QDKSRIIDEF MSQGPTMMVG  600
TcHMA3P   ELKSLGIKTA MLTGDNRDAA MSTQEQLGNA LDIIHSELLP QDKSRIIDEF MSQGPTMMVG  600
AtHMA3    ELKSLGIQTA MLTGDNQDAA MSTQEQLENA LDIVHSELLP QDKARIIDDF KIQGPTMMVG  600

TcHMA3G   DGLNDAPALA KADIGISMGI SGSALATETG DIILMSNDIR KIPRGMRLAR RSHKKVIENC  660
TcHMA3P   DGLNDAPALA KADIGISMGI SGSALATETG DIILMSNDIR KIPRGMRLAR RSHKKVIENC  660
AtHMA3    DGLNDAPALA KADIGISMGI SGSALATETG DIILMSNDIR KIPKGMRLAK RSHKKVIENV  660

HMA3G     VLSVTIKGAI MVVAFAGYPL IWAAVLADAG TCLLVILNSM MLLRDESEVE SACYRAFPSS  720
TcHMA3P   VLSVTIKGAI MVLAFAGYPL IWAAVLADAG TCLLVILNSM MLLRDESEVE SACYRAFPSS  720
AtHMA3    VLSVSIKGAI MVLGFVGYPL VWAAVLADAG TCLLVILNSM ILLRDEREAV STCYRSS-TS  720

TcHMA3G   SPVKLEDDEA QDLEVGLLQK SEETSKKSCG SGCC---KDD QQK 763
TcHMA3P   SPVKLEDDEA QDLEVGLLQK SEETSKKSCG SGCC---KDD QQK 763
AtHMA3    SPVKLEEDEV EDLEVGLLQK SEETSKKSCC SGCCSGPKDN QQK 763
```

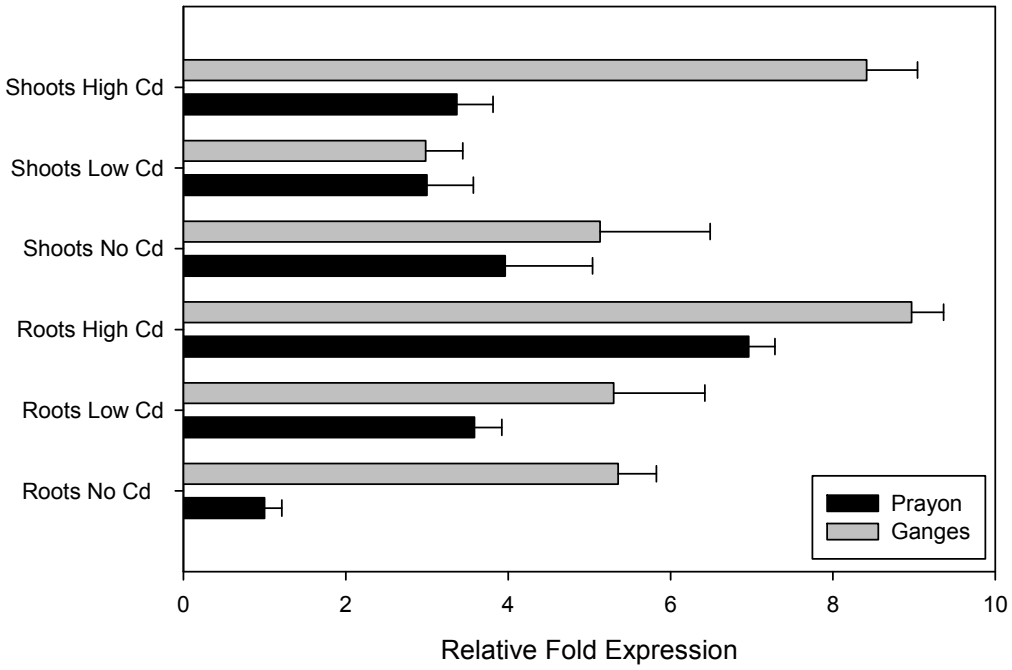

Figure 4.3: Quantitative real time PCR analysis of *TcHMA3* expression in roots and shoots of Prayon and Ganges from replete and Cd grown plants. The average transcript abundance from two biological replicates with SD are shown. Expression of shoots and roots were normalized to actin levels for differences in expression. Prayon root expression under replete conditions was set to one for comparison. Black bars represent Ganges expression, and gray bars represent Prayon expression. Low Cd refers to Prayon and Ganges treated for seven days with 100 µM $CdSO_4$. High Cd refers to Prayon and Ganges plants grown for seven days on 0.5 or 1 mM Cd SO_4 respectively.

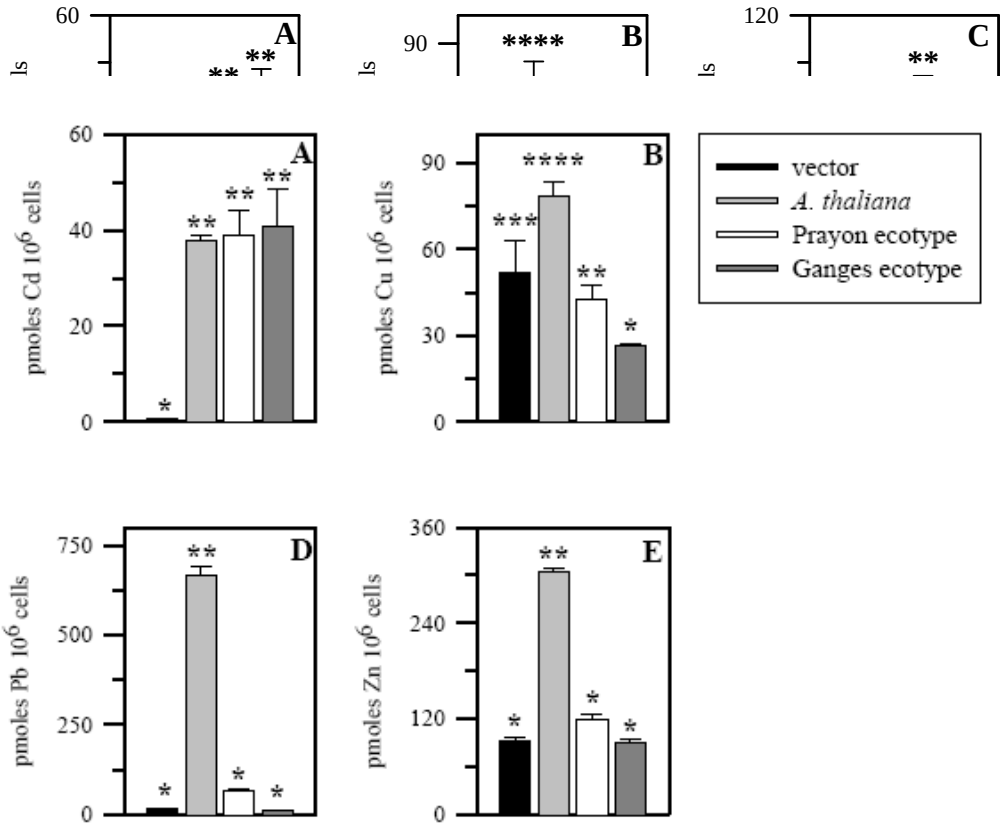

Figure 4.4: Yeast Accumulation of DY1457 cells expressing one of the three orthologs of *HMA3* or empty vector grown with the addition of 10 µM of either Cd, Cu, Pb and Zn for 12 hours and analyzed for mineral content by ICP. One star represents significance less than 0.05, two stars represents significance less than 0.01.

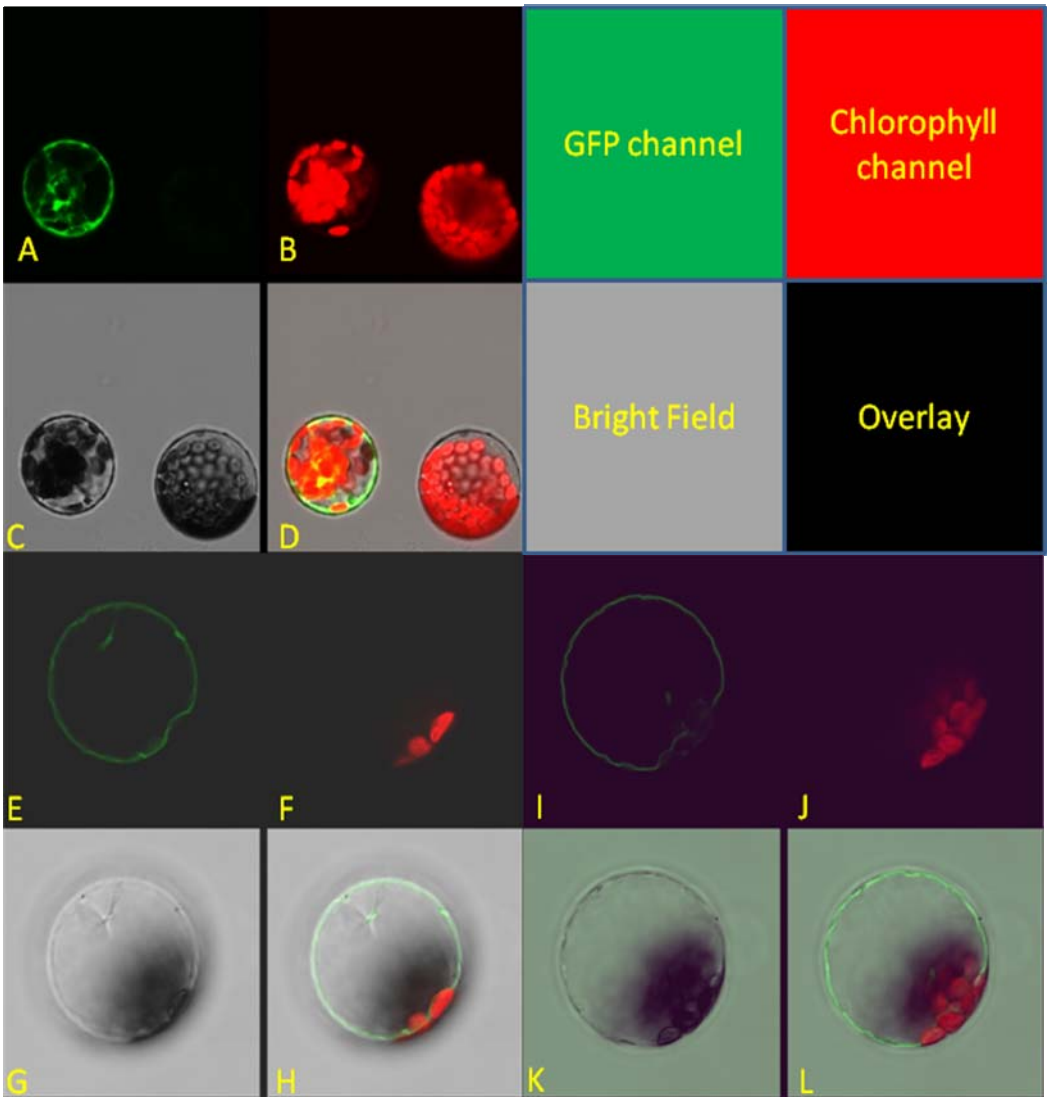

Figure 4.5: Localization of TcHMA3:eGFP fusion protein transiently expressed in Arabidopsis protoplast. Expression was monitored twelve hours after transfection of the protoplast. **A-D**: cytoplasmic eGFP, **E-H**: TcHMA3-P, and **I-L**: TcHMA3-G. The upper left panel is the eGFP signal, the upper right is the autoflorescence of the chloroplast, lower left is the bright field image, and lower right an overlay of all three images.

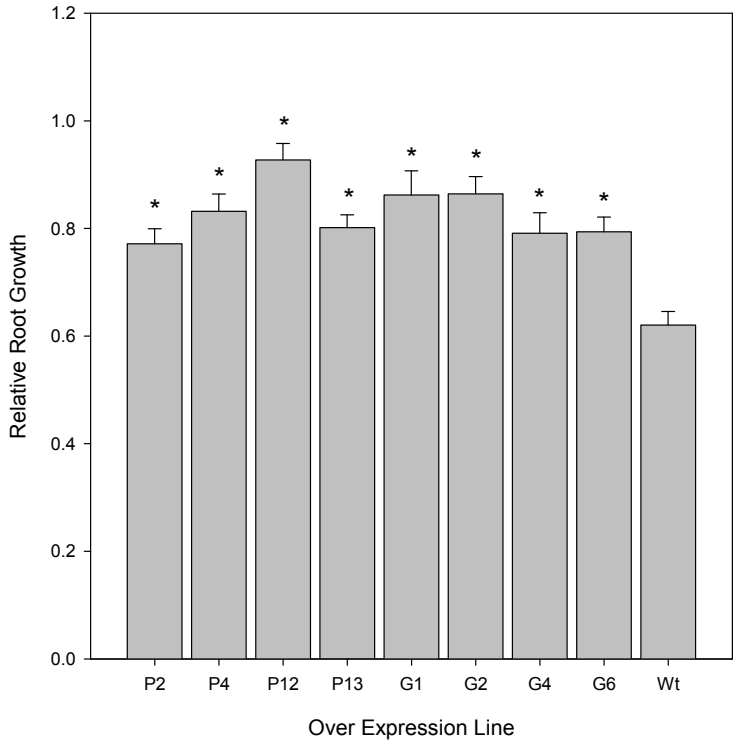

Figure 4.6: Relative root growth of transgenic Arabidopsis plants overexpressing either *TcHMA3-P* or *TcHMA3-G*. Relative root growth is root growth for each transgenic line grown on 20 µM Cd divided by root growth for the same line grown on the same media without Cd. Measurements were taken 14 days after germination. Values are the means with SE (N= 4 replicates). Significance was determined using ANOVA Tukey's post hoc analysis.

expressed either ortholog of *TcHMA3*, the plants showed an increased Cd tolerance compared to Col-0 plants. The top four transgenic lines expressing either ortholog exhibited between a 33 to 56% increase in root growth (relative root growth) under Cd stress compared to Col-0 grown plants (Figure 4.6).

When two of these eight overexpression line expressions were grown on nutrient solution containing 5 μM Cd, roots accumulated roughly 40% more Cd compared to roots of Col-0 plants, while shoot Cd levels were slightly decreased (up to 20%) (Figure 4.7).

As overexpression of the two *TcHMA3* orthologs in Arabidopsis conferred moderate increases in Cd tolerance and accumulation, we thought it would be useful to compare *HMA3* expression levels in WT Arabidopsis, transgenic Arabidopsis overexpression lines, and *HMA3* expression in *T. caerulescens*. We realize that increased expression of any one gene will not confer Cd hyperaccumulation, as the trait is relatively complex and requires the coordinated functioning of a number of different cell types, tissues and organs in *T. caerulescens*. Nevertheless, we thought the comparison of *HMA3* expression would be informative. Hence, we quantified expression of the endogenous *TcHMA3* and *AtHMA3* genes in *Thlaspi* and Arabidopsis (WS ecotype), respectively, and compared this with the expression of the TcHMA3-P and TcHMA3-G in a number of transgenic Col-0 Arabidopsis lines. As seen Figure 8, expression of the *TcHMA3* orthologs in transgenic Arabidopsis were 2 to 8-fold higher than the expression of *AtHMA3* in Arabidopsis, but their expression was still considerably lower than *TcHMA3* expression in *Thlaspi* (*TcHMA3* expression in Prayon was 1.5 to 6-fold higher than transgene expression in Arabidopsis).

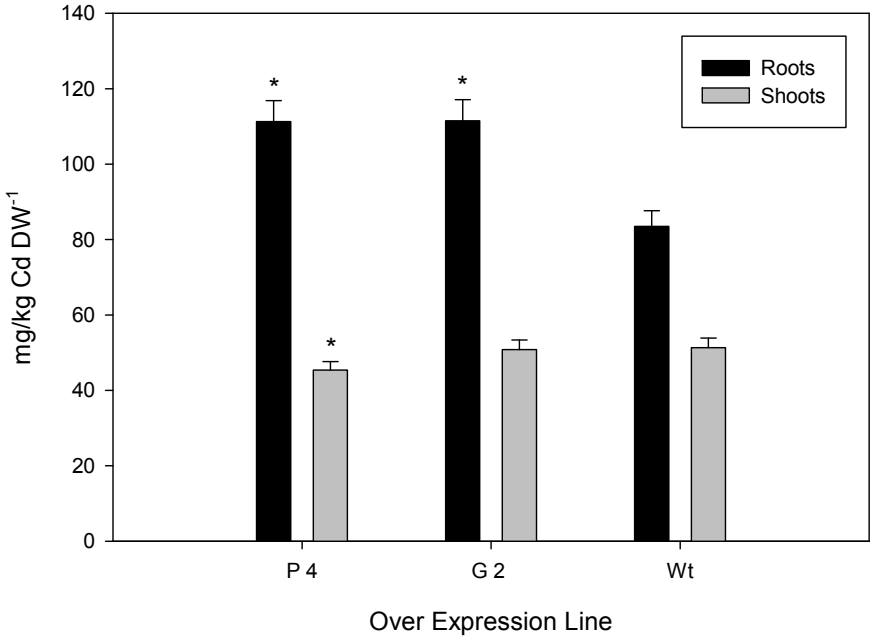

Figure 4.7: Cd content in the roots and shoots of two over expression lines expressing either *TcHMA3-P* or *TcHMA3-G* compared to Col-0 plants grown on 5 µM Cd for seven days. Represented is the means of two biological replicates.

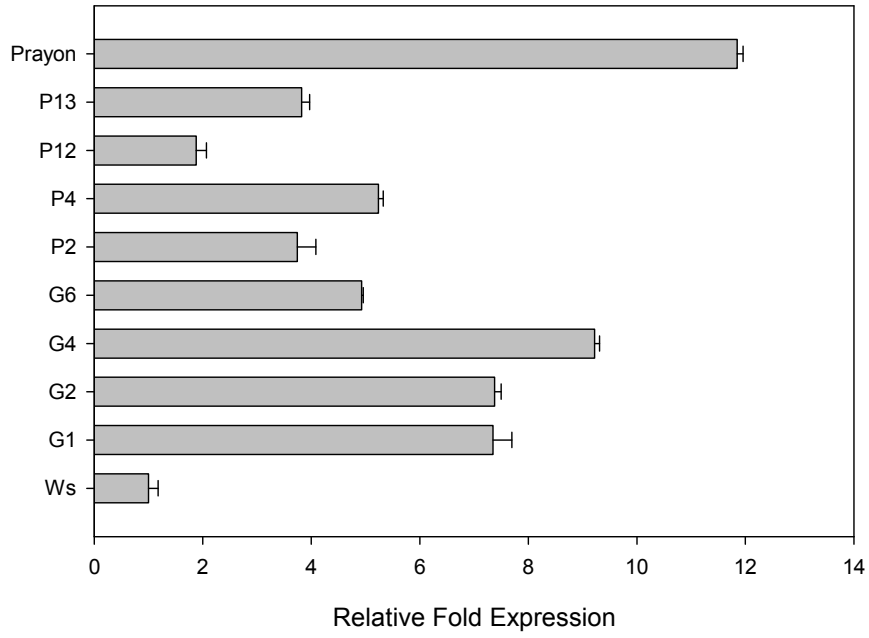

Figure 4.8: Comparison of relative expression levels for *AtHMA3* in wild type Arabidopsis (Ws ecotype), Arabidopsis overexpressing either *TcHMA3* ortholog (G1-P13), and expression of *TcHMA3* in *T. caerulescens* (Prayon) grown on replete nutrient solution. Relative expression levels were determined in reference to the transcript abundance for the *AtHMA3* ortholog in shoots of the Ws ecotype . The relative expression values were normalized by amount of cDNA added to each qPCR reaction and also to actin levels. Arabidopsis Ws expression was set to 1 for comparison.

DISCUSSION

The ability of certain species to take up and accumulate toxic levels of metal is an uncommon trait in the plant kingdom. *Thlaspi caerulescens* is one species which has this unique ability and also demonstrates significant intraspecific variation in metal transport and tolerance. In an attempt to better understand the mechanistic basis of the differences in Cd hyperaccumulation between the Prayon and Ganges populations of *T. caerulescens*, a comparative genome-wide expression approach was taken in shoots of Ganges and Prayon plants exposed to high levels of Cd. The logic behind this approach was to identify shoot genes that were expressed at higher levels in response to Cd stress in Ganges *versus* Prayon. From this comparison, 21 metal transporters were shown to have higher expression in the shoots of Ganges, (Table 4.1). Of these 21 transporters *TcHMA3* stood out as a logical candidate to begin study on, because of the suggested role of AtHMA3 in tonoplast Cd transport (Morel et al, 2009). When plants were treated with increasing levels of Cd (Figure 4.3), *TcHMA3* showed a Cd inducible increase in expression in the roots of Prayon and Ganges. When stressed with even higher Cd levels, Ganges exhibited further increases in *HMA3* expression in both roots and shoots, however in Prayon this additional increase in expression was not seen. This may explain previous reports which have shown an increased Cd tolerance in *T. caerulescens* protoplasts when the plants from which protoplasts were isolated were exposed to Cd (Cosio et al., 2004; Marquès et al., 2004).

Characterization of the *Thlaspi* HMA3 transporters in yeast revealed similar transport characteristics for Cd as compared to the Arabidopsis HMA3 homolog (Figure 3). However, there were significant differences in metal

transport properties in that the *Thlaspi* HMA3 transporters did not transport Cu, Zn, or Pb as well as the Arabidopsis transporter when expressed in yeast. In Arabidopsis Col-0 plants overexpressing *TcHMA3* from either Thlaspi ecotype, there was a distinct increase in Cd tolerance compared to WT Col-0 plants, with an associated increase in root Cd accumulation. (Figures 4.6 and 4.7). It is interesting to note that in transgenic Arabidopsis plants where the transgene is driven by the high expression CaMV35S promoter, *TcHMA3* expression was still considerably lower than the expression of the endogenous *TcHMA3* gene in *T. caerulescens* (Figure 4.8).

The identification of multiple copies of *HMA3* in the *T. caerulescens* genome is in agreement with the hypothesis put forth by Hanikenne et al. (2004) that gene hyperexpression in this plant species is due, in part, to multiple gene copies in the *T. caerulescens* genome. However given the differences in expression seen between the two *T. caerulescens* ecotypes, gene copy number alone does not appear to be the lone determining factor in elevated gene expression in hyparaccumulators.

It is tempting to speculate that *TcHMA3* may be an important component of the Cd hyperaccumulation phenotype in *T.* caerulescens, also playing a role in the ability of specific ecotypes such as Ganges to accumulate higher levels of Cd and not Zn. With regard to Zn hyperaccumulation, which is similar in ecotypes such as Ganges and Prayon, we speculate that the previously identified *T. caerulescens* transporter, *ZTP1* (Assunção et al., 2001), might play a key role in the Zn hyperaccumulation in the two ecotypes. ZTP1 is an ortholog of the Arabidopsis transporter, MTP1, which is a member of the cation diffusion facilitator superfamily of transporters and has been shown to be a vacuolar Zn transporter in Arabidopsis (Desbrosses-Fonrouge

et al., 2005). We found from our microarray analysis that *TcZTP1* exhibits similar levels of expression in both the Prayon and Gange ecotypes, which would be consistent with it playing a role in the similar levels of leaf Zn accumulation seen in these two ecotypes.

To date there have been two relatively low resolution QTL mapping studies for Zn and Cd accumulation in *T. caerulescens* (Assunção *et al*., 2006; Deniau *et al*., 2006). It is interesting to note that in these studies one of the QTLs for root and shoot Cd accumulation were co-localized to the same genomic region, suggesting the same gene is associated with both QTL. *TcHMA3* expression occurs in both roots and shoots under Cd exposure and as shown here, higher levels of *TcHMA3* expression are seen in Ganges. Thus we are intrigued by the possibility that *TcHMA3* may be the underlying gene for this Cd accumulation QTL (Deniau et al., 2006). Additional Cd accumulation QTL mapping studies in a population made between the Zn/Cd hyperaccumulator, *A. halleri*, and the related non-accumulator, *A. lyerta*, suggested that *TcHMA4* may contribute to the ability to accumulate large amounts of Cd in the shoot, as a major shoot Cd accumulation QTL co-localized to *HMA4* (Courbot *et al*., 2007). However, as there are no significant differences in *HMA4* expression between Prayon and Ganges, we feel it is unlikely this gene contributes significantly to the differences seen in Cd accumulation by shoots of Prayon *versus* Ganges.

The Cd inducibility of *TcHMA3*, especially in *Thlaspi* roots, suggests that this gene might also play a role in inducible Cd tolerance in *Thlaspi*, especially in the Ganges ecotype (Küpper et al., 2007; Papoyan et al., 2007). This raises the possibility for the existence of Cd inducible elements in the *TcHMA3* promoter. Also, as seen in Figure 4.3, the Cd induced increases in

TcHMA3 expression occur at higher Cd levels in the superior Cd accumulator, Ganges, compared with Prayon. It will be interesting to determine if this is a cause or effect of the increased Cd accumulation in Ganges.

In summary, a comparative transcriptomics approach was employed here to identify genes more highly expressed in the shoot of the superior Cd accumulating ecotype of *T. caerulescens*, Ganges, compared with the Prayon ecotype. Both ecotypes hyperaccumuate Zn equally well. Using this approach, we identified a metal transporter, TcHMA3 which is a member of the P_{1B}-type ATPase subfamily that is more highly expressed in Ganges shoots and roots. Functional characterization of this transporter both in yeast and *in planta* showed that it is a tonoplast Cd transporter that quite likely plays a key role both in shoot Cd hyperaccumulation and tolerance in general in *T. caerulescens*, and also in the enhanced Cd hyperaccumulation in the Ganges ecotype.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of the Prayon and Ganges ecotypes of *Thlaspi caerulescens* were surface sterilized in dilute bleach (0.5%) and subsequently with 70% ethanol before being germinated in MS plates (MS+vitamins at 4.43 g L^{-}, 0.05% MES at pH 5.7, 1% Sucrose, 1.5% bacto-agar). The plates were placed in a Percival Scientific growth chamber (Model E-36 L, Boone, IA, USA) at a 30º angle. Germinating seeds were illuminated with a combination of fluorescent and incandescent lights at an intensity of ~150 µM m^{-2} s^{-1} with a 16 hr photoperiod and held at ambient humidity with a 24ºC/20ºC day/night temperature cycle. After 17 d of growth, seedlings from plates showing no

contamination were transferred to a hydroponic solution with the following composition: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 µM KCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.1 µM $NiSO_4$, and 0.1 µM H_2MoO_4 (Ebbs *et al.* 2003). The solution was aerated and buffered with 1 mM *n*-morpholinoethanesulfonic acid (MES), titrated to pH 6.0 with KOH. Iron was provided as 10 µM Fe-EDDHA from Sequestrene 138 (Becker-Underwood, Ames, IA). Plants were grown under the same growth chamber conditions as above until plants reached the 8-10 leaf stage (~32 d). Plants of each ecotype were then transferred to the same nutrient solution supplemented with $CdSO_4$ to final concentrations of 0, 0.1, 0.5, 1, 5, or 10 mM. Each treatment was replicated three times. After a 7 d exposure, plant shoots were visually inspected for evidence of heavy metal toxicity (e.g., chlorosis).

Cd treatment for microarray experiment

Seeds of the Prayon and Ganges ecotypes of *T. caerulescens* were germinated and grown as above to the 8-10 leaf stage. Seedlings of Prayon and Ganges were transferred to fresh nutrient solution supplemented with either 0.5 or 1 mM $CdSO_4$, respectively, and subjected to a seven day treatment, also as described above with four replications of each treatment. These concentrations represent the highest concentration from the dose response experiment that did not produce visual symptoms of toxicity in that ecotype. Following the treatment period, shoots were quickly rinsed, patted dry, and split into two subsamples. One subsample was immediately snap frozen in liquid nitrogen, and stored at -80ºC. These samples were shipped by courier on copious amounts of dry ice to the Robert W. Holley Center, Ithaca,

NY. The second subsample was dried at 60ºC to constant mass and ground to <2 mm. These samples were digested following EPA method 3050b (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3050b.pdf) and filtered through a 0.45 µm Millipore filter to remove particulates. The tissue extracts were analyzed for Cd using a SpectrAA 220FS Atomic Absorption Spectrometer (Varian Inc., Walnut Creek, CA). Tissue Cd concentrations in shoots of the Prayon and Ganges ecotypes were compared with the Student's t-test in SPSS (SPSS for Windows, Ver. 13.0).

Microarray experiment

Plants grown and mention above were ground to a fine powder using a mortar and pestle using liquid nitrogen to keep the sample frozen. Total RNA was isolated using the Plant RNeasy RNA mini kit (Qiagen, Valencia, CA) and treated with one unit of DNase Amp. Grade for 30 minutes to remove genomic contamination (Invitrogen, Carlsbad, CA). The protocol was followed as described in the Affymetrix Technical Analysis Manual. This included approximately

One µg of total RNA was reverse-transcribed at 42˚C overnight to generate first-strand cDNA using 100 pmol oligo dT primer using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Following first-strand synthesis, second-strand cDNA was synthesized using *Escherichia coli* polymerase I, *E. coli* DNA ligase and RNase H. The second-strand was incubated at 16˚C for 2 hrs before T4 DNA polymerase was added and the reaction allowed to proceed for an additional five min. The reaction was terminated by adding 0.5 M EDTA. Double-stranded cDNA products were purified using the GeneChip® Sample Cleanup Module (Affymetrix, Santa

Clara, CA). The synthesized cDNAs were *in vitro* transcribed by T7 RNA polymerase using biotinylated nucleotides to generate biotinylated complementary RNAs (cRNAs). The cRNAs were purified using the GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA). The cRNAs were then fragmented at 94˚C for 35 min to generate molecules of approx. 35–200 bp. *Arabidopsis thaliana* ATH1 GeneChip® arrays (Affymetrix, Santa Clara, CA) were hybridized with 15 µg of fragmented labelled cRNA for 16 h at 45˚.

The genomic DNA (gDNA)-based probe-selection strategy described in Hammond et al. (2006) was used to process our transcriptome dataset. The CEL files generated by hybridizing *T. caerulescens* genomic DNA samples to Arabidopsis ATH1 GeneChip® arrays were obtained from http://affymetrix.arabidopsis.info/xspecies. These gDNA CEL files were used to mask the probes in the Arabidopsis ATH1 array Chip Description File (CDF) with a gDNA hybridization intensity threshold of 300, using the Xspecies perl script (Hammond et al., 2006). The probe-masked CDF file was used to process and normalize *T. caerulescens* RNA CEL files at probe level with the RMA algorithm (Irizarry et al., 2003). The detection calls (present, marginal, or absent) for each probe set were obtained using the mas5calls function in the Affy package (Gautier et al., 2004). Only genes with at least one present call across all the compared samples were used to identify differentially expressed genes. Significance of gene expression was determined using the LIMMA test (Smith, 2005) and raw p values of multiple tests were corrected using the False Discovery Rate (FDR, Benjamini and Hochberg, 1995). Genes with FDR < 0.05 were identified as differentially expressed genes.

Quantitative Real Time PCR

Transcript abundance was assayed for in *T. caerulescens* plants either Prayon or Ganges ecotypes using plants grown for two weeks in the nutrient solution described above. The plants were split into two groups, with one half placed in fresh nutrient solution and the second half in fresh nutrient solution amended with 100 µM Cd. After a one week exposure to Cd, the plants were harvested and snap frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Total RNA was isolated using the Plant RNeasy RNA mini kit (Qiagen, Valencia, CA). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Transcript levels were measured using GoTaq® qPCR Master Mix (Promega, Madison, WI) using the primer pair TTGATTTCTCCGTTATCGTCGTT and ACGATTTGAAGCGGAGATATCAAG. Actin was used as an internal reference amplified using the primer pair GAAGAACTACGAGCTACCTGATG and GATCCTCCGATCCAGACACTGTA. Quantitative real-time RT-PCR was performed using an ABI 7500 real-time PCR system and SYBR Green kit (Applied Biosystems). PCR conditions used were 95 C for 5 min followed by 40 cycles of 95 C for 30 sec, 47.9 C for 30 sec and 60 C for 1 min. A thermal dissociation curve was performed after each of the two biological replicates to ensure only one product was being amplified. A subsample of the product of the qRT-PCR reaction for each ortholog of HMA3 and actin was further amplified by PCR, cloned into the pGEM easy T vector (Promega, Madison, WI), and sequenced for target verification.

Cloning of HMA3

Primers were designed based on sequence homology between the rice

and Arabidopsis HMA3 genes to amplify a 1.1 kb region of the coding region using the primers ACCGTCATCGTTGTCCAC and ATCGGATGGCTTGACTTG. RACE was then performed to clone the full 5' and 3' ends of the gene in Prayon (Clonetech RACE kit, Mountain View, CA). The primers CTAATACGACTCACTATAGGGCTGCAGTGG and CCACTGCAGCCCTATAGTGAGTCGTATTAG were then used to isolate the putative open reading frame from Prayon and cloned into pGEM easy T vector for sequence verification. The same primers were then used to amplify the ORF from Ganges.

Yeast culture and transformation

Each ortholog of TcHMA3 was digested from the pGEM easy T vector used to verify sequence with *NotI* and cloned into a linearized pFL61 opened with *NotI*. The correct orientation of the insert was verified by PCR using the primer pair TCTCGCTTCTTGCCATACTATTGCTTTTGA and TTAAAATACGCTGAACCCGAACATAGAAAT. A plasmid containing *AtHMA3* was obtained from Dr. Pierre Richaud, CEA Cadarache and the ORF was amplified using the primer pair CAAGCTCAACGATGGCGGAAGGTG and CAGAAGAAGGTTTTCACTTTTG and cloned into pGEM easy T vector. This vector was digested with *NotI* and the fragment obtained cloned into pFL61 at the *NotI* restriction site. The primer pair CAAGCTCAACGATGGCGGAAGGTG and TTAAAATACGCTGAACCCGAACATAGAAAT was used to identify colonies bearing this construct and sequencing was conducted to confirm that the sequence was inserted in the correct orientation. *Saccharomyces cerevisiae* strain DY1457 (MATα *ade6 can1 his3 leu2 trp1 ura3*) was obtained from Dr.

David Eide (University of Wisconsin) and cultured on YPD plates (per liter: 10 g yeast extract, 20 g bacto-peptone, 20 g glucose, 15 g bacto-agar). Transformation of this strain with the target construct (pFL61 vector and the pFL61 vector with the gene of interest) was performed using the lithium acetate/single-stranded carrier DNA/PEG method (Gietz and Schiestl 2007). To obtain cells for transformation, a single colony was streaked on a fresh YPD plate and incubated for ~2 d at 30ºC. Cells scraped from this plate were suspended in 1.0 mL of sterile deionized water and then pelleted by centrifugation (13,000*g* for 30 sec). The supernatant was discarded and the following, in sequence, were layered over the pellet: 240 µL PEG 3350 (50% w/v), 36 µL 1.0 M lithium acetate, 10 µL single-stranded carrier DNA (10 mg mL^{-1}, herring sperm DNA boiled for 5 min to cause denaturation), and plasmid DNA (0.5 – 1 µg), and sufficient sterile deionized water to provide a final volume of 360 µL. The mixture was vigorously vortexed for up to 1 min and subjected to a heat shock at 42ºC for 20 min. For some constructs, the transformation mix was held overnight at room temperature to enhance the transformation efficiency (Gietz and Schiestl 2007). The transformation mix was then centrifuged at 13,000*g* for 30 sec to pellet the cells. After the supernatant was decanted, the cells were resuspended in 1.0 mL of sterile deionized water. Aliquots of the resuspended cells were plated onto a synthetic complete uracil dropout selection media (referred to hereafter as SC-URA media). The SC-URA media contained per liter: 6.7 g yeast nitrogen base with ammonium sulfate, without amino acids, 2 g synthetic complete amino acid supplement minus uracil, 20 g glucose, 0.1 g adenine sulfate, 15 g bacto-agar. Plates were incubated for 3-5 d at 30ºC until transformants were

observed. Single colonies were picked from each transformant plate and established on fresh SC-URA plates.

Metal uptake by yeast transformants

Single colonies from SC-URA plates were cultured with shaking in liquid SC-URA media at 30ºC until the cells reached an OD_{600} of ~1.0. An aliquot of this primary culture was used to establish a subculture with fresh SC-URA media at an OD_{600} of 0.1. The media was supplemented $CdSO_4$, $CuSO_4$, $ZnSO_4$, or $Pb(CH_3COO)_2$ at a final concentration of 10 µM. Each metal treatment was replicated five times and each experiment was performed at least twice to confirm the results. The subcultures were cultured with shaking at 30ºC for 12 hr. The OD_{600} was recorded and cell number in the culture media was determined. The cells were pelleted by centrifugation at 3,000*g* and washed three times with sterile deionized water. After the final wash the cells were resuspended in ~2.0 mL of sterile deionized water and stored at -20ºC. To prepare the cells for elemental analysis, the tubes were thawed and the contents transferred to clean glass tubes. The cell suspension was heated to 95ºC and 1.0 mL of concentrated nitric acid (Trace Metal Grade) was added to facilitate lysis of the cells. The cell suspension was heated to 95ºC, held for 20 min, and then subjected to three alternating cycles of 45ºC for 5 min and 95ºC for 20 min. This temperature regime was repeated following the addition of 1 mL of 30% hydrogen peroxide. The cell lysate was diluted to a final volume of 10 mL with 5% nitric acid. The concentration of the metal of interest in the lysate was determined using a 220FS atomic absorption spectrometer (Varian Inc, Walnut Creek, CA) and the results were normalized to the cell number. For each metal, the comparison of the means used a one-way

ANOVA in SPSS for Windows (Ver. 13.0) with Tukey's test for post hoc analysis.

Plant Transformation

The plant expression vector pBAR was used to over-express either a *TcHMA3* construct. The TcHMA3 constructs were subcloned into pBAR using EcoRI and BamHI restriction sites added to the 5' and 3' ends, respectively, via PCR. The two different pBAR constructs containing the different HMA3 genes were transformed into *Agrobacterium tumefaciens* line C58 and selected for on Kanamycin plates. Agrobacterium-mediated transformation of Arabidopsis ecotype Columbia (Col-0) was performed using the floral dip method (Clough and Bent, 1998; modified from Bechtold et al. 1993).

Metal tolerance and accumulation in *TcHMA3* over-expression lines

Seeds of the Prayon and Ganges ecotypes of *Thlaspi caerulescens* were surface sterilized in dilute bleach (0.5%) and subsequently with 70% ethanol before being imbibed in 0.1% low melting point agarose and kept at 4˚C for five days. Seeds were then sown out on the same modified Johnsons solution plus or minus 20µM $CdSO_4$. Plants were grown for 14 days after germination weeks under the growth chamber conditions described above. Total root length of each plant were determined using Root Reader 2D (www.plantmineralnutrition.net). Relative root length was the calculated by dividing the mean total root length of the Cd-treated plants by the mean total root length of the control plants. The data were compared using a one way ANOVA with Tukey's test for post hoc analysis. To determine mineral content, plants were grown for two weeks in a replete Johnsons solution. After

two weeks plants were treated with 5 μM $CdSO_4$ for seven days. Dry weights were determined and the samples were analyzed by ICP-AES for Cd content. To determine significance in Cd content, the comparison of the means used a one-way ANOVA with Tukey's test for post hoc analysis.

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CHAPTER V

FUTURE WORK

INTRODUCTION

The research detailed in this thesis has focused on mechanisms and regulation of metal transport process underlying heavy metal and micronutrient hyperaccumulation and homeostasis in *Thlaspi caerulescens*. This research has implications for the phytoremediation of heavy metal contaminated soils. But from a broader point of view, the work detailed here that also addresses micronutrient homeostasis in plants is a starting point for a slightly different line of research looking at how the plant cell senses its mineral nutrient requirements, which occurs at multiple levels from the level of the cell all the way to the functioning of the intact plant. However, the molecular and physiological basis for mineral nutrient homeostasis is still poorly understood in plants. Additionally, almost nothing is known about the linkages between the control of plant mineral nutrient acquisition and fundamental processes such as cell division and growth.

Micronutrient deficiencies, and particularly zinc (Zn) and iron (Fe) deficiency, are the most important nutritional deficiency problems for both plants and humans in both developing and industrialized countries. As much as 30% of the world's soils are considered Zn deficient with large areas in developed and developing countries including China, India, Mexico, and the US all suffering from low Zn soils. This results in decreases in both crop yield and Zn accumulation in edible portions of plants. A better understanding of the uptake of Zn and Fe from the soil and their translocation and accumulation

in consumed portions of the plant is necessary for providing important fundamental information for improving both food security and quality.

FUTURE WORK

E2F's

With the ability of the two TcE2F's to restore growth to the *zap1Δ* yeast mutant under Zn limiting conditions, the work reported here addressed the ability of these plant transcription factors to activate expression of a yeast high affinity Zn transporter. The concern we had was that this finding may have been an artifact that was *Thlaspi* specific for this particular yeast mutant. To test if E2F involvement in regulation of mineral nutrient transporters is specific for *T. caerulescens*, we first looked at whether E2Fs from other plant species could also rescue the *zap1Δ* yeast mutant when grown on Zn limiting conditions. Three Arabidopsis E2F genes AtE2F1, AtE2F2, and AtE2F3, were cloned in pFL61 and transformed in the *zap1Δ* yeast mutant. When yeast cells expressing any of these three Arabidopsis E2F genes were grown under Zn limiting conditions, all three E2F family members were able to rescue the mutant and allow increased growth (Figure 5.1). With the ability of the Arabidopsis E2F's to also complement the *zap1Δ* yeast mutant, we now have the ability to investigate this topic in a plant species with a sequenced genome and a full range of genetic resources to further address if the E2F family can regulate micronutrient transporters.

The current assumption is that TcE2F1 and TcE2F2 have the ability to bind to the promoter of the high affinity Zn transporter *ZRT1* and activate expression. While this was not been directly shown in this thesis, we did show that *ZRT1* transcripts were seen when either TcE2F1 or TcE2F2 was expressed in the

zap1Δ yeast mutant but not in yeast expressing the empty pFL61 vector. ZRT1 is a member of the ZIP family of micronutrient transporters, and our recent preliminary findings showing putative activation of *ZRT1* by members of the Arabidopsis E2F family of proteins, this opens up a broader screen to look at interactions between Arabidopsis E2Fs and all the members of the ZIP family of transporters in Arabidopsis to identify potential binding sequences and activation using yeast as a heterologous system. A preliminary scan of 1 kb upstream of the transcription initiation site for each of the 15 Arabidopsis ZIP family members revealed that 10 of the 15 members contain at least one putative E2F element

in the promoter. But an essential experiment to run before we get too far into the characterization of the E2F family would be to show that activation of *ScZRT1* is through binding of the E2F protein to a promoter element. To show that the *ScZRT1* can be activated by any of the five E2F proteins cloned so far, the promoter of *ScZRT1* has been cloned upstream of a LacZ reporter and will be co-expressed with each of the E2F proteins to test for activation. This same system will be used for testing of each of the ZIP promoters for activation by the five different E2F proteins cloned so far.

If activation of a ZIP family member cannot be found, the CASTing assay, or *C*yclic *A*mplification and *S*election of *T*argets, will be employed with the three different AtE2F proteins that complemented the *zap1Δ* yeast mutant, to identify the E2F binding sequence for each E2F protein (Wright et al, 1993). A genome wide scan will then be performed to find targets for each of the AtE2F proteins.

Proteins involved in Zn uptake and identification of a zinc responsive element

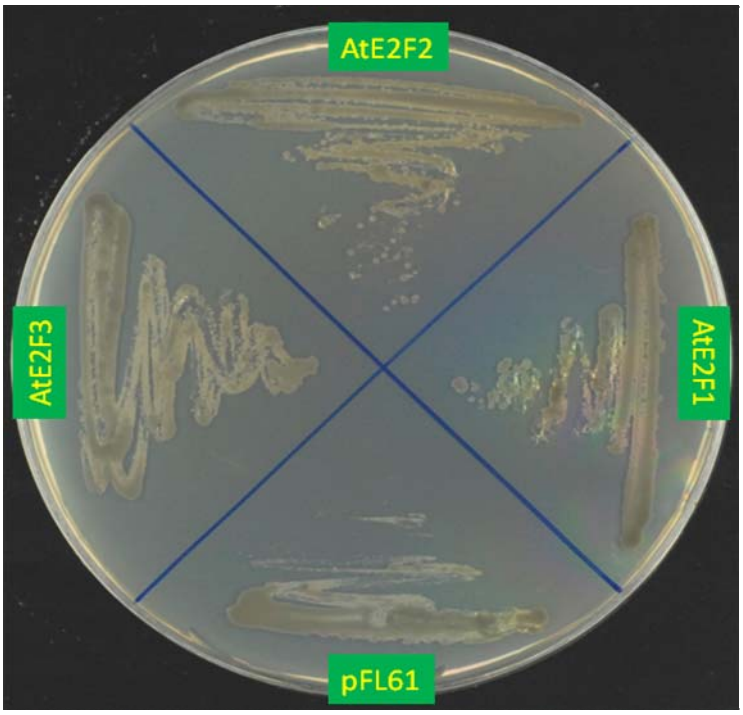

Figure 5.1: Complementation of the growth phenotype of the *zap1Δ* by three members of the AtE2F family of proteins. Yeast cells transformed with *AtE2F1, AtE2F2, AtE2F3* or pFL61 vector control were streaked out on Zn limiting media (SC-URA + 1mM EDTA + 500 μM $ZnSO_4$) and grown for 72 hours to check for growth.

The role and function of the different members of the ZIP micronutrient family of transporters is still not understood. A case in point is AtZIP4, which because of its sequence homology to TcZNT1 was assumed to play a role in root Zn transport. However, its localization to the chloroplast it remains unclear what role AtZIP4 plays in plant mineral nutrition. One of our future goals is to identify which AtZIP transporters are involved in uptake from the soil. Therefore I have cloned thirteen of the fifteen transporters from the Arabidopsis ZIP family and will be used to first determine the membrane localization of each ZIP member using transient expression in Arabidopsis protoplast. Those showing plasma membrane localization in protoplast will be expressed in yeast to characterize their transport capabilities. qPCR has already been performed on these thirteen genes and eleven of the transporters are expressed in the roots, and five of the eleven ZIPs show higher expression in the roots than in shoots.

For these thirteen members of the ZIP family I already have also cloned the one kb upstream of the transcription initiation site, have made promoter::GUS reporter fusions and have stably transformed each construct into transgenic Arabidopsis seedlings to identify in which specific tissues these genes are expressed and test for activation of each gene by Zn, Cu, Mn and Fe. From these experiments the members of the ZIP family which are expressed in the root epidermis and or root cortex and the protein is localized to the plasma membrane, I will test homozygous T-DNA knockout lines for those genes tested for their susceptibility or tolerance to metals. Their ability to complement known uptake mutants in yeast will also be examined. For the *in planta* work, I have isolated homozygous T-DNA lines for each ZIP family member except for AtZIP8 where the T-DNA insertion is located either within

the coding region or within 1 kb of the coding region on either the 5' or 3' side. Seven of the thirteen genes have insertions in an exon, and the rest have insertions in either the 5' or 3' UTR's or an intron. These lines will be screened for sensitivity to each of the metals shown to be transported in yeast. If no phenotypes are seen, it may be necessary to generate double and triple knockouts for different ZIPs in order to obtain a phenotype.

Results from these experiments should provide a wealth of data about the metal-regulated expression of each of these genes. Subsequently, a comparative sequence approach in conjunction with functional analysis of sequential promoter deletions should allow for the identification of metal responsive elements in the promoters of specific ZIP family members. The strength of studying the promoters of multiple genes whose expression is Zn responsive will help us quickly to identify candidate DNA sequences in the promoters that may play a role in the regulation of expression in response to changes in Zn status that will be the focus of more in detail study. We are also interested in regulation of gene expression by other micronutrients such as Cu. With regards to Cu, a Cu responsive element has been identified in the promoter of several Arabidopsis genes (Quinn and Merchant, 1995) and we identified this element in the promoters of *AtZIP2*, *AtZIP3 and AtZIP4*.

Our major goal is to identify cis acting elements for Zn responsiveness. As reported in Chapter 4, roughly one kb upstream of the transcription initiation site was enough to drive expression of either *TcZNT1* or *AtZIP4* under Zn limiting conditions. Five prime deletions were made for both the *TcZNT1* and *AtZIP4* promoters regions removing increments of roughly 100 bp for each deletion of both the *TcZNT1* and *AtZIP4* promoters and stably transformed into Arabidopsis to test for Zn deficiency.

Previous work from a former member of the Kochian lab had identified a putative palimndromic Zn responsive element based on analysis of the Arabidopsis and *T. caerulescens ZIP* promoters for ZIPs known to be Zn responsive, using the multiple expectation-maximization for motif elicitation or MEME analysis software, which is a probability matrix-based algorithm (http://www.sdsc.edu/MEME). . This sequence: ATGTCGACAT or ATGTCGACAC, is found in the promoters of both *TcZNT1* and *AtZIP4*. These palindromes are located between -226 and -308 from the translational initiation site in the ZNT1 promoter and between -30 and +88 in the *AtZIP4* genomic region. Scanning the other thirteen ZIP family members reveals a degenerate palindromic version of this sequence in six other ZIP family members (Pence 2002). This putative ZRE sequence will help direct a portion of our promoter deletion studies. If a *bona fide* ZRE is identified, it can be used in yeast one hybrid screens with an Arabidopsis a cDNA library to begin to search for other trans acting factor(s) regulating ZIP expression.

Cd and Zn accumulation

With the identification of *TcHMA3* as a potential player in Cd accumulation in both the roots and shoots of *T. caerulescens*, and the apparent lack of involvement of HMA3 in Zn transport and accumulation suggests that different genes are involved in the Zn and Cd hyperaccumulation phenotypes. A previously identified gene in Arabidopsis, *ZAT1*/*MTP1,* has been shown to transport Zn and confer tolerance to high levels of Zn in the media when expressed both in yeast and Arabidopsis (Van der Zaal et al., 1999; Delhaize et al., 2003). While the homolog of *AtMTP1*, *TcMTP1/TcZTP1* which was identified based on sequence homology to

AtMTP1, has been suggested to be involved in the loading of Zn into the vacuole, no direct experimental evidence for this has been presented (Assunção et al., 2001; Mäser et al., 2001, Persans et al., 2001). We have cloned *TcMTP1/TcZTP1* from the Prayon ecotype of *Thlaspi* and will express a TcMTP1 in yeast to study its transport properties; additionally, overexpression of *TcMTP1* in Arabidopsis will help to better define this gene's role in hyperaccumulation and Zn and Cd tolerance. It is tempting to speculate that *TcMTP1* plays a key role in the Zn hyperaccumulation phenotype while *TcHMA3* is involved in the Cd hyperaccumulation phenotype as discussed in Chapter 3.

CONCLUSIONS

The future research detailed in this chapter will help to further define the roles of the ZIP and E2F families of proteins in plant micronutrient homeostasis. These two families remain poorly characterized in plants and it is likely they have broad roles in a number of different plant processes.

If we determine that the E2F family of transcription factors plays a role in the regulation of the nutritional needs of the plant in response to abiotic environmental stresses, this will significantly advance our understanding of how the cell perceives and regulates its nutrient requirements. The proposed future research should identify those members of the Arabidopsis E2F family, long associated with cell division, that are also involved in micronutrient homeostasis. Furthermore, the research detailed here should identify some of the mineral nutrition-associated target genes for these E2F family members. These studies will contribute needed fundamental information for generating novel approaches for engineering crop species with the enhanced capacity to

acquire Zn from the soil and also accumulate Zn and possibly other essential micronutrients in grains and other edible tissues in the plant. Hence, this work has significant implications for both plant and human nutrition, due to the widespread worldwide occurrence of both Zn deficient soils and Zn deficiency in humans.

A better understanding of the transport of micronutrients into and around the plant should result from the proposed characterization of the ZIP family of transport proteins. While several ZIP family members have been crudely characterized and for their transport abilities, to date a comprehensive investigation of this transporter family has not been conducted. Finally, the identification of micronutrient responsive elements in the promoters of ZIP family members will lead to a better understanding of the regulation and control of the different ZIP transporters shed light on how the different members of this family function to maintain the delicate balance between deficiency and toxicity for a number of essential micronutrients.

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